# Raffinose Family Oligosaccharides (RFOs) Are Putative Abiotic Stress Protectants: Case Studies on Frost Tolerance and Water Deficit in *Ajuga reptans* and *Arabidopsis thaliana*

---

**Dissertation**

**zur**

**Erlangung der naturwissenschaftlichen Doktorwürde**

**(Dr. sc. nat.)**

**vorgelegt der**

**Mathematisch-naturwissenschaftlichen Fakultät**

**der**

**Universität Zürich**

**von**

**Shaun W. Peters**

**aus**

**Süd Afrika**

**Promotionskomitee**

**Prof. Dr. Enrico Martinoia (Vorsitz)**

**Prof. Dr. Felix Keller (Leitung der Dissertation)**

**Dr. Norbert Sprenger**

**Zürich, 2010**

**SUMMARY**

Raffinose family oligosaccharides (RFOs) are $\alpha$1,6 galactosyl extensions of sucrose, occurring exclusively in plants and some photoautotrophic algae. They have important roles as phloem translocates and carbon stores in plants. Additionally, correlative mass increases in RFOs during abiotic stress events have been observed. Despite such correlations, an *in vivo* functional role for RFOs in abiotic stress tolerance remains speculative as the stress tolerance response represents a concerted multigenic phenomenon that results in the accumulation of a number of other molecules with predicted roles in abiotic stress protection (e.g. sucrose and proline).

As part of a multipronged strategy to further examine the role/s of RFOs in abiotic stress tolerance, we performed the following experiments.

(i) A previously described excised leaf system from the frost hardy evergreen labiate, *Ajuga reptans*, which effectively uncouples RFO accumulation from low temperature-acclimation was refined by physiologically validating that the basic carbohydrate metabolism of excised leaves is similar to that of whole plants. Using classical $^{14}C$ photosynthetic pulse/chase experiments, we demonstrated that excised leaves operate within normal photosynthetic parameters producing $^{14}C$-sucrose as the major photosynthate and transporting $^{14}C$-stachyose as the main phloem translocate, comparable to previous published data obtained with whole plants. Furthermore, in excised leaves, RFOs increased in both concentration and oligomer length over time. Using excised leaves grown in the warm with low, intermediate and high RFO concentrations, we demonstrated that frost tolerance (as determined by $EL_{50}$, the temperature at which 50% electrolyte leakage occurred) increased from about -10.5°C to -24.5°C as RFO concentrations increased

(ii) Using Arabidopsis *atgols1* and *atgols1 atgols2* T-DNA loss-of-function mutants [galactinol synthases (GolS) are responsible for the first committed step in RFO biosynthesis; *AtGolS-1* and *-2* are water deficit-induced], we demonstrated that both soil-droughted mutants underwent a loss of leaf relative water content (RWC) more rapidly than wild type (Col-0) plants. The mutants showed a visible loss of leaf turgor at 53-60% RWC when Col-0 plants remained fully turgid (80% RWC). The typical water deficit-induced increase in GolS activity was absent in both *atgols2* and *atgols1 atgols2* plants. Galactinol and raffinose accumulation decreased strongly in stressed

leaves of the mutant plants. Surprisingly, in stressed mutant leaves, sucrose accumulation decreased to less than 50% of stressed Col-0 leaves. An Arabidopsis raffinose synthase T-DNA loss-of-function mutant was also reported to display attenuated sucrose accumulation during cold acclimation. These data suggest that water-soluble carbohydrate accumulation in Arabidopsis during abiotic stress events is a complex process, possibly a homeostatic balance between a number of osmolytes. Whilst our data clearly demonstrates that GolS activity is a prerequisite for water deficit-induced accumulation of galactinol and raffinose, it is unclear whether the hypersensitive phenotype is the result of perturbation of raffinose accumulation alone or a combined effect of both sucrose and raffinose accumulation.

(iii) To manipulate RFO concentrations in Arabidopsis while keeping the RFO biosynthetic pathway intact, the sequences of known and functionally expressed alkaline α-galactosidases (α-Gals, enzymes involved in the first step of RFO hydrolysis) were compared against the Arabidopsis gene database. Two candidate genes (*ATSIP1* and *-2*) were found. Since *ATSIP1* had been previously characterised and predicted to be involved in RFO biosynthesis, we focused on *ATSIP2* which we predicted to possibly encode an α-Gal. When *ATSIP2* was heterologously expressed in Sf9 insect cells, crude cell lysates hydrolysed raffinose, contrary to uninfected control cells. Recombinant ATSIP2 enzyme hydrolysed, from the artificial substrate, *p*-nitrophenyl-D-galactopyranoside, only the α- and not the β-form, displayed a pH optimum of 7.5-8.0 and was inhibited by deoxygalactonojirimycin (a classical α-Gal inhibitor) as well as high galactose concentrations. No raffinose synthase activity was observed. Collectively, these results unequivocally demonstrate that ATSIP2 is an alkaline α-Gal. This finding is important in the context of recent publications which present real time PCR data for *ATSIP2* during abiotic stress and define it as a raffinose synthase. Using this newly discovered α-Gal we created an overexpression vector (pMDC32::*ATSIP2*) and transformed Arabidopsis with it. We provide preliminary and partial proof-of-concept data demonstrating that, in independent T1 transgenic lines, *ATSIP2* is overexpressed and alkaline α-Gal activity is higher than in untransformed wild type plants.

**ZUSAMMENFASSUNG**

Raffinose Familie Oligosaccharide (RFOs) sind α1,6 Galactosyl-Verlängerungen der Saccharose, die ausschliesslich in Pflanzen und einigen photautotrophen Algen vorkommen. Sie spielen eine wichtige Rolle als Phloemzucker und Kohlenhydratspeicher. Zusätzlich wird oft eine positive Korrelation zwischen RFO Zunahme und abiotischen Stressereignissen beobachtet. Trotz dieser Korrelation bleibt eine *in vivo* Funktion der RFOs bei abiotischer Stresstoleranz spekulativ, da die Entwicklung der Stresstoleranz ein konzertiertes Phänomen ist, an dem mehrere Gene beteiligt sind und mehrere andere Moleküle (z.B. Saccharose und Prolin) akkumulieren können.

Als Teil einer breitgefächerten Strategie, um die Rolle(n) der RFOs bei abiotischer Stresstoleranz weiter zu erforschen, führten wir folgende Experimente durch.

(i) In einer Weiterentwicklung des früher beschriebenen Systems mit abgeschnittenen Blättern der frosttoleranten, immergrünen Labiate *Ajuga reptans* konnte gezeigt werden, dass die RFO-Akkumulation von der Akklimatisierung bei tiefen Temperaturen abgekoppelt werden kann und der Grundkohlenhydratstoffwechsel ähnlich verläuft wie der von ganzen Pflanzen. In klassischen $^{14}$C-Pulse/Chase Photosynthese-Experimenten zeigte sich, dass sich abgeschnittene Blätter physiologisch innerhalb normaler Photosynthese-Parameter bewegen mit $^{14}$C-Saccharose als Haupt-Photosyntheseprodukt und $^{14}$C-Stachyose als Haupt-Phloemzucker. Dieses Verhalten war ähnlich wie das von ganzen Pflanzen in der Literatur beschriebene. In abgeschnittenen Blättern nahm in der Wärme sowohl der RFO Gehalt als auch die RFO Kettenlänge mit der Zeit zu. Versuche mit abgeschnittenen Blättern mit tiefem, mittlerem und hohem RFO-Gehalt zeigten, dass die Frosttoleranz mit zunehmendem RFO-Gehalt von -10.5°C auf -24.5°C anstieg (bestimmt als $EL_{50}$, die Temperatur, bei der die Blätter 50% ihrer Elektrolyte verloren hatten).

(ii) Mit Arabidopsis *atgols1* und *atgols1 atgols2* T-DNA Loss-of-function Mutanten [Galactinol Synthasen (GolS) katalysieren den ersten Schritt der RFO Biosynthese; *AtGolS-1* und *-2* sind durch Wassermangel induziert] zeigten wir, dass bei Bodentrockenheit bei beiden Mutanten der relative Blatt-Wassergehalt (RWC) schneller abnahm als beim Wildtyp (Col-0). Die Mutanten zeigten einen sichtbaren Verlust des Blatt-Turgors bei 53-60% RWC, während die Col-0 Pflanzen turgeszent blieben (80% RWC). Der sonst bei Wassermangel übliche Anstieg der GolS Aktivität blieb in beiden *atgols1* und *atgols1 atgols2* Mutanten aus. Die Galactinol und

Raffinose Akkumulation in gestressten Blättern beider Mutanten war gehemmt. Überraschenderweise zeigten die Blätter der gestressten Mutanten eine um mehr als 50% reduzierte Saccharose Akkumulation verglichen mit denjenigen der gestressten Col-0 Pflanzen. Eine Arabidopsis Raffinose Synthase T-DNA Loss-of-function Mutante hatte früher ebenfalls während der Kälteakklimatisierung gehemmte Saccharose Akkumulation gezeigt. Diese Daten deuten an, dass die Akkumulation von wasserlöslichen Kohlenhydraten bei abiotischem Stress ein komplexer Prozess ist, möglicherweise ein homeostatisches Gleichgewicht einer Anzahl von Osmolyten. Während unsere Resultate klar zeigten, dass die GolS Aktivität eine Voraussetzung für die Akkumulation von Galactinol und Raffinose ist, ist noch unklar, ob der überempfindliche Phänotyp ausschliesslich die Folge einer gestörten Raffinose Akkumulation oder ein kombinierter Effekt der Saccharose und Raffinose Akkumulation ist.

(iii) Um die RFO Konzentration in Arabidopsis unter Beibehaltung des RFO Biosyntheseweges zu manipulieren, wurden zunächst die Sequenzen bekannter und funktionell exprimierter alkalischer α-Galactosidasen (α-Gal, Enzyme des ersten Schrittes der RFO Hydrolyse) gegen die Arabidopsis Gendatenbank verglichen. Die beiden Kandidatengene, *ATSIP1* und *-2,* wurden dabei gefunden. Da früher schon *ATSIP1* charakterisiert und als der RFO Biosynthese zugehörig vorgeschlagen worden war, konzentrierten wir uns auf *ATSIP2* und vermuteten, dass es sich dabei um eine α-Gal handeln könnte. Heterologe Expression von *ATSIP2* in Sf9 Insektenzellen lieferte ein Zelllysat, das Raffinose hydrolysierte (im Gegensatz zum Lysat nicht infizierter Zellen). Rekombinantes ATSIP2 Enzym hydrolysierte ferner vom künstlichen Substrat, *p*-Nitrophenyl-D-Galactopyranosid, nur die α- und nicht die β-Form, zeigte ein pH Optimum von 7.5-8.0, wurde vom klassischen α-Gal Hemmstoff Deoxygalactonojirimycin und hohen Galactosekonzentrationen gehemmt. Raffinose Synthase Aktivität wurde keine gefunden. Insgesamt zeigen diese Resultate eindeutig, dass ATSIP2 eine alkalische α-Gal ist. Dieses Ergebnis ist wichtig im Zusammenhang mit neuesten Publikationen, die *ATSIP2* Echtzeit-PCR Daten von abiotischen Stressexperimenten präsentieren und dabei von *ATSIP2* als Raffinose Synthase ausgehen. Mit dieser neu entdeckten α-Gal konstruierten wir einen Überexpressionsvektor (pMDC32::*ATSIP2*) und transformierten damit Arabidopsis Pflanzen. Vorläufige Resultate zeigten, dass *ATSIP2* in unabhängigen T1 transgenen Linien überexprimiert ist und die dort gemessene alkalische α-Gal Aktivität höher ist als in untransformierten Wildtyp Pflanzen.

# Chapter I: General Introduction

# GENERAL INTRODUCTION

## 1.1 The abiotic stress syndrome

Plants are sessile organisms and, therefore, do not possess the “flight” reactions commonly associated with organisms challenged with adverse environmental conditions. Consequently, plants have developed a remarkable capacity to counteract the deleterious effects of both biotic and abiotic stresses. Despite various general responses to stress, the mechanisms underpinning these responses may be grouped into three categories in plants viz. escape, avoidance and tolerance mechanisms (Altman, 2003; Vinocur and Altman, 2005). Whilst escape strategies are physiologically valid, they represent specific life histories where plants such as desert ephemerals germinate and reproduce rapidly when water is available, surviving prolonged periods of drought as seeds. Avoidance mechanisms mainly result from morphological and physiological changes at the whole-plant level (e.g. succulence, leaf folding) whilst tolerance mechanisms are the result of extensive stress-induced cellular and molecular biochemical modifications, making the latter particularly attractive to biotechnological manipulation (Wang et al., 2001).

## 1.2 Abiotic stresses manifest common physiological effects

As a critical component of metabolism, water enables many vital biological reactions by being a solvent, a transport medium, an evaporative coolant as well as a medium and reactant in biochemical reactions (Bohnert et al., 1995). In plants and other photoautotrophs, water plays the additional role of providing the electrons necessary to drive photosynthesis. It is thus unsurprising that some abiotic stresses (e.g. water deficit, high salinity and freezing) can elicit similar responses, manifesting themselves as physiological dehydration at the cellular level (Vinocur and Altman, 2005). This is due to the unavailability of water at the sub-cellular level. It stands to reason that if osmotic stress is a common feature of different abiotic stresses then stress-induced gene expression should reveal an overlap of genes transcriptionally upregulated by these stresses, particularly if these genes are involved in osmotolerance.

Extensive analysis of changes in the Arabidopsis transcriptome during exposure to various abiotic stresses using microarray technology has demonstrated that genes induced by stress can be categorized into two groups according to the functions of their products. (Bohnert et al., 2001; Seki et al., 2001; Zhu et al., 2001). The first group (Figure 1.1) is comprised of proteins with putative functional roles in stress protection such as membrane proteins that maintain water and solute movement

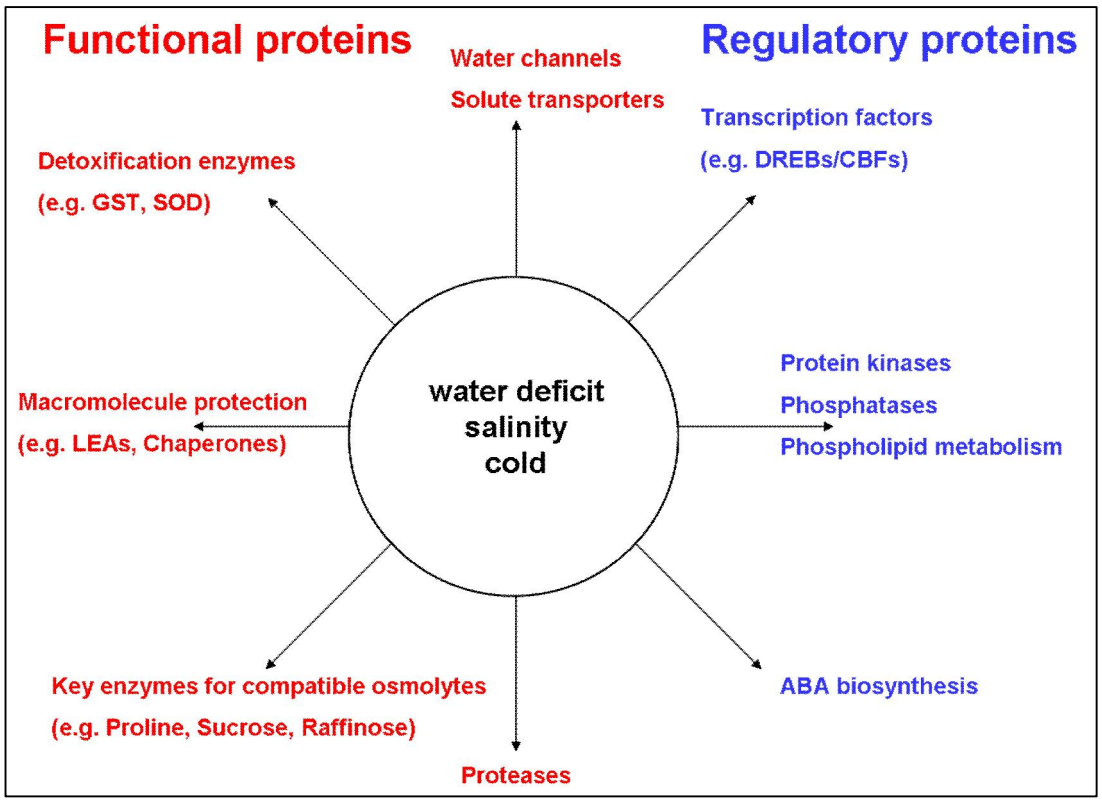


**Figure 1.1**: Categories of stress-inducible genes in Arabidopsis. Proteins with putative function in stress protection are outlined in red (functional proteins), whilst proteins which function in the regulation of signal transduction and gene expression (regulatory proteins) are outlined in blue. Adapted from Shinozaki and Yamaguchi-Shinozaki (2007).

through membranes (water channel proteins and membrane bound solute transporters), key enzymes for compatible osmolyte biosynthesis (e.g. proline, sugars), detoxification enzymes that ameliorate oxidative stress (e.g. glutathione *S*-transferase, superoxide dismutase, ascorbate peroxidase), and other proteins for the protection of macromolecules (e.g. late embryogenesis abundant (LEA) proteins, chaperones).

The second group is representative of proteins thought to have regulatory roles in stress response and includes transcription factors (bZIP, MYC, MYB and DREB, etc.), protein kinases (MAP kinase, CDP kinase, receptor protein kinase, ribosomal protein kinase and transcription-regulation protein kinase, etc.) and proteinases (phosphoesterases and phospholipase C, etc.) involved in the regulation of signal transduction and gene expression.

Transcription factors (TFs) are responsible for the most basic level of gene regulation, modulating gene expression at the level of transcription. They are usuallly

defined as having specific functional domains that bind DNA (DNA binding domains, DBDs) that recognise specific consensus sequences in the promoter regions of the genes they regulate. Extensive analysis of the Arabidopsis genome has identified more than 2000 genes that contain DBDs and are thought to be TFs (Iida et al., 2005; Riano-Pachon et al., 2007). A small family of Arabidopsis TFs has been intensively studied because they are primarily responsible for gene regulation during abiotic stress, in this plant.

### 1.3 DREB/CBF transcription factors orchestrate stress-related gene expression

The C-repeat-binding factor (CBF)/dehydration-responsive element-binding factor (DREB1) proteins constitute a small family of Arabidopsis transcriptional activators (CBF1/DREB1B, CBF2/DREB1C, CBF3/DREB1A and CBF4/DREB1D) that play a prominent role in cold acclimation (Liu et al., 1998; Novillo et al., 2007). Together with the water deficit responsive DREB2 (Liu et al., 1998), they are arguably the most well-characterized family of abiotic stress-responsive TFs to date and represent a clear distinction between stress-induced gene expression, isolating cold stress from water deficit and high salinity. All DREB/CBF transcription factors contain an EREBP/AP2 DNA binding domain (Liu et al., 1998) and recognise the highly conserved C-repeat (CRT) or drought responsive element (DRE), (G/A)CCGAC (Yamaguchi-Shinozaki and Shinozaki, 1994). This element occurs as a *cis* element in the promoters of genes known to be up-regulated by abiotic stresses.

The phytohormone abscisic acid (ABA) plays a central role in abiotic stress related signal transduction mediating many, but not all, responses (Wasilewska et al., 2008). Analyses of the influence of ABA on the regulation of DREB transcription factors concluded that the DREB1 and -2 transcription factors operate independently of ABA (Figure 1.2) (Shinwari et al., 1998; Medina et al., 1999; Nakashima et al., 2000). Conversely, CBF4/DREB1D was demonstrated to be ABA responsive (Haake et al., 2002). Over-expression of DREB transcription factors in rice, Arabidopsis and tobacco resulted in the transcriptional activation of many genes commonly associated with abiotic stress and stress tolerant transgenic plants (Kasuga et al., 2004; Maruyama et al., 2004; Chen et al., 2008), firmly placing this family of transcription factors as key orchestrators of stress related gene expression.

When DREB transcription factors are overexpressed in Arabidopsis, a number of organic osmolytes have been reported to accumulate in vegetative tissue under

normal growing conditions including, proline, sucrose, raffinose, glycine betaine and sinapoyl malate (Mattana et al., 2005; Chen et al., 2008; Maruyama et al., 2009). A common feature of these organic osmolytes is that they have all been implicated to function as stress protectants.

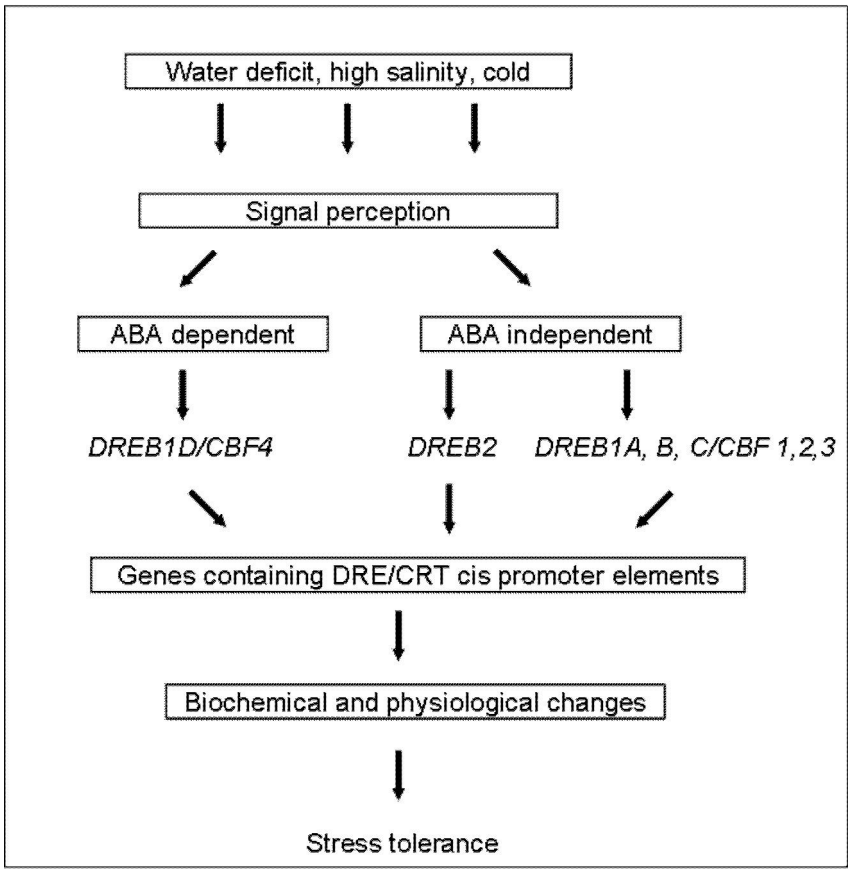


**Figure 1.2**: Schematic representation of events occurring during abiotic stress between signal perception and gene expression. DREB2 distinguishes water deficit and high salinity signal transduction pathways from cold. Genes containing the DRE/CRT *cis* element in their promoters are transcriptionally up-regulated by the DREBs and accumulation of their gene products lead to stress tolerance. Adapted from Agarwal et al. (2006).

### 1.4 Abiotic stress induces accumulation of organic osmolytes

Analyses of stress-induced changes in the Arabidopsis transcriptome and overexpression of specific stress-induced transcription factors has revealed that abiotic stress elicits multi-genic responses, leading to complex biochemical and physiological changes in plant cells.

One of the most ubiquitous responses to abiotic stress, in many unrelated organisms, is an increase of intracellular organic osmolytes. The basic concepts of the regulation and function of these compounds are already well described in a classical review (Yancey et al., 1982). These concepts remain valid and are summarised as follows.

(i) All osmotically stressed organisms, with the exception of halobacteria, accumulate organic osmolytes, (ii) all organic osmolytes are grouped into a few chemical categories including water-soluble carbohydrates (e.g. trehalose, sucrose, raffinose family oligosaccharides), polyols and their derivatives (e.g. glycerol, sorbitol, pinitol), amino acids (e.g. glycine, proline, taurine) and their derivatives (e.g. ectoine) and methylamines (e.g. glycine betaine) and methylsulfonium solutes (e.g. urea, dimethylsulfonopropionate), (iii) stress-induced organic osmolytes accumulate to high concentrations without modifying the functional efficiency of cellular metabolism (for a current review see Burg and Ferraris, 2008).

Historically, it has been thought that these organic osmolytes, often termed compatible solutes, increase in response to osmotic stress in prokaryotes (e.g. hyper-saline environments), thereby raising the internal osmotic pressure of the cell and counteracting the stress (Cushman, 2001). However stress-related increases in compatible solutes have been reported in biological systems (e.g. plants) where concentrations are too low to justify an osmotic effect and thus the functional roles of these solutes have been expanded to include free radical scavenging (Shen et al., 1997; Nishizawa et al., 2008), membrane protection (Galinski, 1993; Hincha et al., 2003) and protein stabilisation (Carpenter et al., 1990; Papageorgiou and Murata, 1995).

The protective efficacy of compatible solutes, their compatibility with cellular metabolism and the advent of targeted molecular engineering implies that unique solutes may be engineered into organisms that do not normally accumulate the solute, thereby introducing constitutive tolerance to abiotic stress. Indeed, many examples of such compatible solute engineering are evident in plants. It has been reported that engineering the ectoine biosynthetic pathway into tobacco resulted in hyperosmotic tolerance in cultured tobacco cells (Nakayama et al., 2000). Ectoine is known to occur only, but ubiquitously, in halotolerant bacterium (Martin et al., 1999). Similarly expression of the *ggpPS* gene for glucosylglycerol biosynthesis, from the halotolerant bacterium *Azotobacter vinelandii* in Arabidopsis, improved salt tolerance in transgenic plants (Klahn et al., 2009). Other examples, using compatible solute biosynthetic genes from prokaryotes, and reporting enhance abiotic stress tolerance in transgenic plants include glycine betaine (Hayashi et al., 1998; Holmstrom et al., 2000; Huang et al., 2000), proline (Nanjo et al., 1999; Hong et al., 2000), mannitol (Tarczynski et al., 1992; Thomas et al., 1999) and trehalose (Holmstrom et al., 1996; Han et al., 2005). Simply put, such a definitive body of evidence supports the notion

that hyperaccumulation of a compatible solute, by default, leads to enhanced abiotic stress tolerance.

The major focus of this thesis is on a group of non-reducing water-soluble carbohydrates termed raffinose family oligosaccharides (RFOs) which have shown to function as typical compatible solutes *in vitro* (Hincha et al., 2003; Cacela and Hincha, 2006) and have been reported to increase in the vegetative tissues of many plant species upon exposure to abiotic stress.

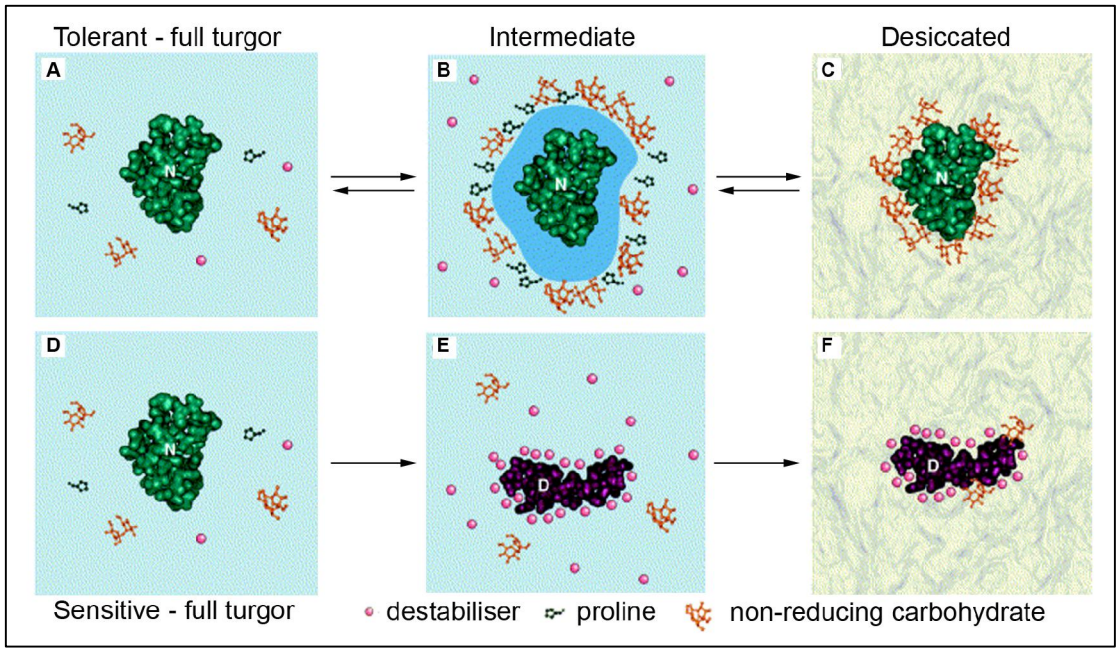


**Figure 1.3**: Proposed protective mechanism of compatible solute accumulation during water deficit. Molecular crowding during water loss increases the probability of cytoplasmic solutes interacting with protein surfaces. In tolerant cells (A-C), the presence of compatible solutes (proline and/or carbohydrate) effectively excludes destabilising molecules from binding proteins. In the desiccated (air-dried) state, sugar molecules effectively replace the water hydration shell, hydrogen bonding to proteins and stabilizing the native protein structure in the dried (glassy) cytoplasm. In sensitive cells (D-F) the lack of compatible solutes causes destabilising molecules to bind proteins, unfolding and denaturing them. The reversibility of the processes occurring during dehydration and rehydration is indicated by arrows. Adapted from Hoekstra et al. (2001).

## 1.5 Raffinose family oligosaccharides (RFOs) are synthesized by α-galactosyl transferases

RFOs (Suc-$[Gal]_n$, $13< n \geq 1$) are α1,6-galactosyl extensions of sucrose (Suc), that occur frequently in higher plants. The RFO biosynthetic pathway is initiated with the synthesis of the galactosyl donor galactinol (Gol; 1-O-α-D-galactopyranosyl-L-*myo*-inositol), catalysed by the enzyme galactinol synthase (GolS, EC 2.4.1.123) using UDP-Gal and *myo*-inositol as substrates. Subsequently, the α-galactosyltransferase raffinose (Raf) synthase (RafS, EC 2.4.1.82) transfers a galactosyl moiety from Gol to the $C_6$ position of the glucose (Glc) moiety in Suc forming an α1,6-galactosidic linkage to yield the trisaccharide Raf. Similarly, stachyose (Sta) synthase (StaS, EC 2.4.1.67) transfers a galactosyl moiety from Gol to the $C_6$ position of the Gal moiety in Raf to yield the tetrasaccharide Sta. In *Coleus blumei* and *A. reptans*, a Gol-independent biosynthetic pathway has been reported for higher RFO oligomer biosynthesis (Bachmann and Keller, 1995; Inan-Haab and Keller, 2002; Tapernoux-Lüthi et al., 2004). Extensive biochemical characterisation of this enzyme in *A. reptans* has revealed that galactan:galactan galactosyl transferase (GGT) utilises RFOs as galactosyl donors and acceptors during chain elongation, facilitating the synthesis of high DP RFO oligomers (up to Suc-$[Gal]_{13}$) in *A. reptans*. Furthermore a novel vacuolar sorting determinant that targets GGT to the vacuole has been identified (Tapernoux-Lüthi et al., 2007)

Similarly, it has been shown that a cDNA isolated from developing pea seeds and functionally expressed encodes a multi-functional StaS, able to catalyse the Gol-independent biosynthesis of both Raf and Sta. Interestingly, the enzyme also displayed RFO hydrolase activity but was unable to initiate RFO biosynthesis using Suc and Gol as substrates (Peterbauer et al., 2002).

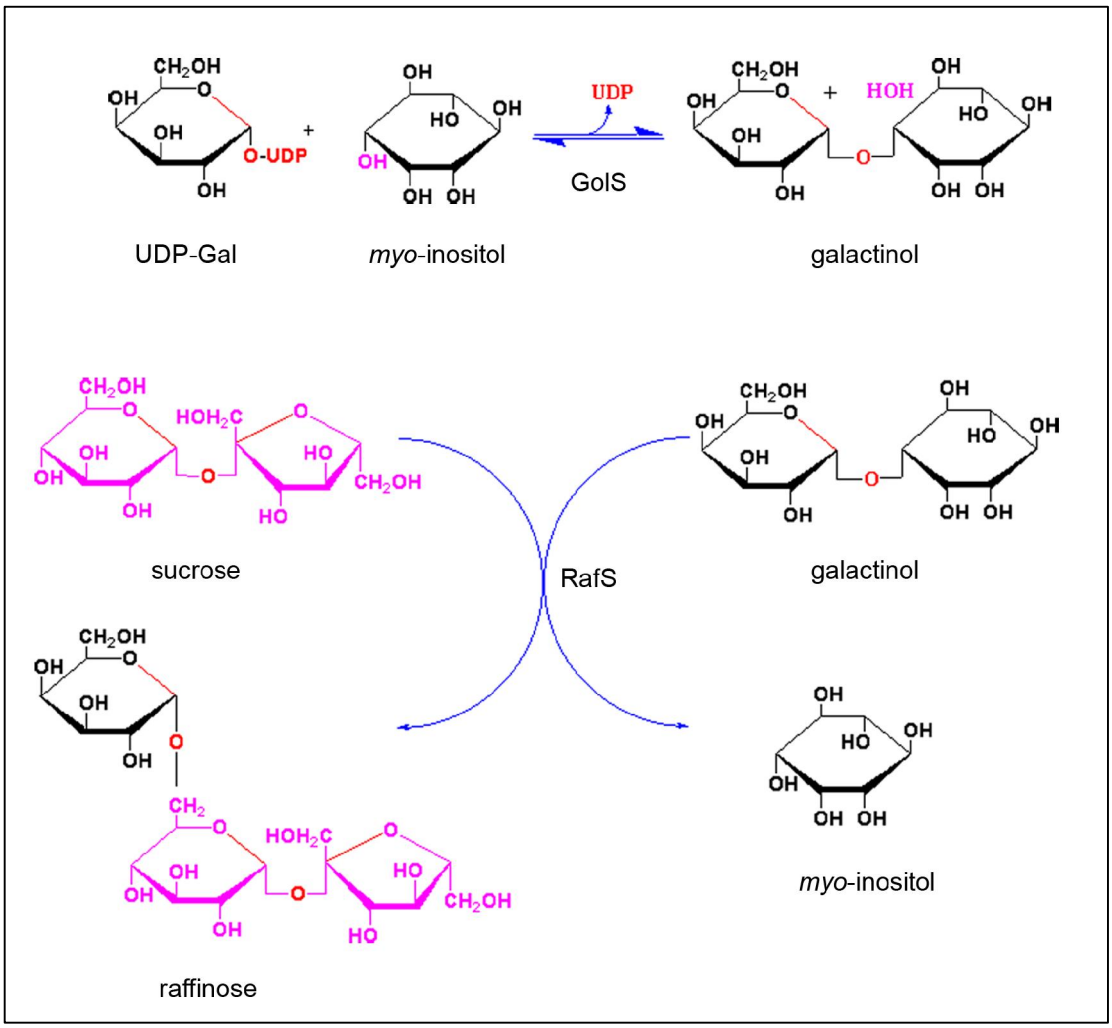


**Figure 1.4**: Biosynthetic pathway of the lowest RFO oligomer, Raf. Gol is synthesised by GolS using UDP-Gal and *myo*-inositol as substrates. The terminal Gal moiety in Gol is transferred to the C6 position of the Glc moiety in Suc, by RafS, to form Raf. Similarly, the stepwise transfer of a Gal moiety from Gol to $C_6$ position of the Gal moiety in Raf, by StaS, yields the tetrasaccharide stachyose. Adapted from www.uky.edu.

Together with Suc, RFOs have been well characterised as having functional roles in carbon translocation and storage in a number of plant families (Zimmerman and Ziegler, 1975; Keller and Pharr, 1996). Whilst the occurrence of RFOs is common in plants, there are exceptional cases where certain species such as *Stachys sieboldii* tubers (Japanese artichoke) and *Ajuga reptans* leaves and roots (common bugle) have been reported to store large quantities of RFOs (Keller, 1992; Bachmann et al., 1994).

### 1.6 RFOs are catabolised by glycosyl hydrolases: α-galactosidases (α-Gals)

Of the numerous families of glycosyl hydrolases, three (27, 36 and 110) are classified as α-Gals (Henrissat, 1991; Henrissat and Romeu, 1995). Whilst families 27 and 36 contain enzymes from both bacterial and eukaryotic organisms, family 110 is composed exclusively of bacterial enzymes but evidently they are represented ubiquitously across the taxonomic kingdom ranging from Archaebacteria to humans (www.cazy.org). The α-Gals (EC 3.2.1.22 α-D-galactoside hydrolase) catalyse the hydrolytic cleavage of the terminally linked α-Gal moieties from Gal-containing molecules and may thus be separated functionally by their physiological roles (sub-cellular compartmentation), substrate specificities and pH optima.

Bacterial and fungal α-Gals often present broad spectrum substrate specificities and are physiologically linked to enhanced utilisation of alternative carbon sources for growth (e.g. Raf and melibiose, de Vries et al., 1999). In humans, the α-Gal A gene is responsible for the catabolism of specialised lipids (glyco-sphingolipids) which contain galactose moieties. Genetic mutations in this gene manifest as Fabry's disease, a debilitating life threatening condition.

Plant α-Gals from a variety of species have been identified, and numerous forms of the enzyme described (for review, see Keller and Pharr, 1996). They may be divided into two groups, based on their pH optima (acid or alkaline). Many studies have dealt with acidic isoforms, which appear to play important roles in seed development and germination (Keller and Pharr, 1996) but a group of functionally unidentified seed imbibition proteins (SIP) were also observed in germinating barley seed embryos (Heck et al., 1991). Subsequently, the cloning and functional expression of two cDNAs from melon fruit showed that they displayed distinct α-Gal activity at alkaline pH. Most importantly, these genes showed highest homology to SIP genes, suggesting that SIP proteins are likely to represent alkaline α-Gals in plants and revealing a previously unknown family of glycosyl hydrolases (Carmi et al., 2003).

They were subsequently identified, on the basis of sequence homology, to be present in at least four other plant families including Poacea (barley, SIP: M77475), Leguminoseae (Cicer, SIP: X95875), Solanaceae (tomato, SIP: TC94379), and Cruciferae (Arabidopsis, SIPs: AAC83062, CAB66109). The two SIP isoforms in Arabidopsis (AtSIP1 and 2) are as yet functionally unclassified and only one relevant study has looked at an *AtSIP1* loss-of-function mutant in the context of water deficit

tolerance, indirectly concluding it to be involved in RFO biosynthesis (Anderson and Kohorn, 2001).

The functional roles of plant α-Gals include (i) the mobilisation of galacto-mannans (specialised carbohydrates located in the seed integument of legumes) during germination (Edwards et al., 1992), (ii) mobilisation of RFOs during seed germination (Blöchl et al., 2008), (iii) cell wall loosening and expansion via hydrolysis of galacto-rhamans linkages (Chrost et al., 2007), (v) galacto-lipid degradation during leaf senescence (Fialho and Bücher, 1995; Thompson et al., 1998) and (v) potential roles in phloem unloading in plants that use RFOs as phloem translocates (Smart and Pharr, 1980; Gaudreault and Webb, 1983; Bachmann et al., 1994; Gao and Schaffer, 1999).

### 1.7 Do RFOs function in abiotic stress tolerance?

*In vitro* studies point to a protective role of RFOs as they have been shown to (i) stabilise artificial liposomes during lyophilisation by hydrogen bonding to the headgroups of the phospho lipid bilayer (Hincha et al., 2003), (ii) enhance cryopreservation of mouse spermatozoa (Tada et al., 1990), (iii) provide enhanced lyo-protection to liposomes with increasing oligomer length (Cacela and Hincha, 2006) and (iv) indirectly reduce the effects of oxidative stress via potential radical scavenging (Nishizawa *et al.*, 2008).

There is a large body of correlative evidence citing significant mass increases of RFOs (particularly Raf) in the vegetative tissue of plants subjected to a variety of abiotic stresses that include low temperature, water deficit, high salinity, heat, and oxidative stress (Santarius, 1973; Bachmann et al., 1994; Taji et al., 2002; Pennycooke et al., 2003; Panikulangara et al., 2004; Peters et al., 2007; Nishizawa et al., 2008; Peters and Keller, 2009). Collectively, these observations suggest that RFOs may be important in stress protection.

However, *in vivo* observations taken in the context of multi-genic responses to abiotic stress in plants have led to contradictions in the functional significance of RFOs in stress tolerance. A recent comparative analysis of Arabidopsis T-DNA insertion mutants for RafS (Raf deficient) and transgenic plants overexpressing a cucumber GolS, revealed no significant differences in the stress performance of these plants

when they were subjected to cold acclimation and freezing (Zuther et al., 2004). Such discrepancies will be discussed in the following sections.

### 1.7.1 Orthodox seeds are desiccation tolerant and contain RFOs

RFOs are found abundantly in desiccation tolerant orthodox seeds and are often absent or detectable in trace quantities in desiccation sensitive recalcitrant seeds (Lin and Huang, 1994; Sun et al., 1994). In orthodox seeds representing many different species, RFO accumulation has been shown to coincide with the onset of desiccation tolerance during the maturation stage of seed development (Koster and Leopold, 1988; Leprince et al., 1993; Horbowicz and Obendorf, 1994; Gorecki et al., 1997; Zhu et al., 2007). It has been suggested that seed desiccation tolerance in maize seeds cannot be achieved in the absence of RFOs but that low concentrations of RFO do not necessarily impart desiccation tolerance (Brenac et al., 1997). Desiccation tolerance in soybean seeds strongly correlates to RFO content (Blackman et al., 1992). There are conflicting reports suggesting that RFOs are not a prerequisite at all for desiccation tolerance in cauliflower seeds (Hoekstra et al., 1994).

Other reports promulgate a function for RFO accumulation in seeds as readily metabolisable carbon storage sources which are utilised during the germination process (Downie and Bewley, 2000). In support of this, germination in pea seeds has been demonstrated to be dependent on RFOs. The results of that study showed that germination of pea seeds was delayed by several days when $\alpha$-Gal activity (and thus RFO catabolism) was inhibited by the $\alpha$-Gal inhibitor 1-deoxygalactonojirimycin (DGJ, Blöchl et al., 2007). Conversely, similar experiments conducted for soybean seeds failed to replicate this delay in germination concluding that, at least for soybean, RFO mobilisation is not critical for seed germination (Dierking and Bilyeu, 2009).

In Arabidopsis, quantitative trait loci (QTL) studies did not reveal any correlation between QTL for seed storability and Suc or Raf content (Bentsink et al., 2000; Clerkx et al., 2004). The obvious lack of consensus between the results of the studies described warrants questioning the assumed functional importance of RFOs in seed physiology. Many reports suggest that it is the ratio of RFO-to-Suc in mature seeds that is of critical importance to desiccation tolerance, and not the absolute concentrations of these sugars (Koster and Leopold, 1988; Brenac et al., 1997; I. S. Carvalho, 2005; Zhu et al., 2007). It has thus been proposed that Suc is the major protectant in mature seeds but that Raf is essential, in order to prevent Suc

crystallisation in the mature, anhydrobiotic state of mature orthodox seeds. It has been reported from *in vitro* studies that Raf is able to physically adsorb to the growing crystal face of Suc, thereby disturbing the formation of Suc crystals (Mantovani et al., 1988; Campañá Cué et al., 2001). Such an observation has direct biological relevance in anhydrobiotic systems such as seeds and dessication tolerant plants, where Suc crystallisation during desiccation would cause membrane damage.

Collectively, such observations do not necessarily discount a role for sugars in seed desiccation tolerance and perhaps in the contradictions described, there is less reliance on sugar content and more on other factors for tolerance in these species.

### 1.7.2 RFO accumulation in vegetative tissue correlates to stress tolerance

Correlative mass increases in RFOs have been reported in vegetative and generative tissue of a number of plant species in response to water deficit (Brenac et al., 1997; Black et al., 1999; Pattanagul and Madore, 1999; Peters et al., 2007), low temperature (Imanishi et al., 1998; Cunningham et al., 2003; Konrádová et al., 2003), high salinity (Gilbert et al., 1997; Riccardo et al., 1998; Jouve et al., 2004; Sanchez et al., 2008), heat shock (Panikulangara *et al.*, 2004) and oxidative stress (Nishizawa *et al.*, 2008) suggesting that they may function as a general stress protectant in plants.

#### 1.7.2.1 Are RFOs necessary during desiccation of vegetative plant tissue?

A small group of angiosperms (about 330 species) has been reported to display true desiccation tolerance (Proctor and Pence, 2002). These plants, termed resurrection plants, display a unique phenomenon amongst higher plants of being truly desiccation tolerant. As such they are ideal models to study the physiology of desiccation tolerance since they remain viable in the anhydrobiotic state by maintaining a metabolic stasis, and recover fully within 72 h of rewatering (Farrant, 2000; Collet et al., 2003; Vicré et al., 2004).

Sugars, in particular Suc, would appear to accumulate universally in the leaves of many different resurrection plant species during water deficit (Bianchi et al., 1993; Müller et al., 1997; Ghasempour et al., 1998; Albini et al., 1999; Zivkovic et al., 2005; Peters et al., 2007) and as such Suc is thought to be the primary carbohydrate involved in the desiccation tolerance responses of angiosperm resurrection plants. However, in some of the resurrection plants there are water deficit-induced increases

in the disaccharide trehalose and RFOs, but never to the same extent as Suc (Farrant, 2007).

We have recently demonstrated significant mass increases of both Suc and RFOs in the leaves of the monocotyledonous resurrection plant *Xerophyta viscosa*, during desiccation. This was the first report where the Suc-to-Raf mass ratio (1.3:1) in leaves exceeded a very typical ratio (never lower than 5:1) of other resurrection plants (Peters et al., 2007). Similarly to observations made in seeds, we have suggested that mass ratios of different sugars and not necessarily their absolute concentrations, contribute to any protective properties exerted in desiccated tissues. In the absence of full genome sequences and reliable transformation methods for resurrection plants however, such observations are at best good correlations between carbohydrate mass increases (Suc, Raf) and desiccation tolerance and fail to address the functional significance of these sugars in tolerance.

In a pioneering study, Taji et al. (2002) provided some molecular evidence that the constitutive overexpression of *AtGolS2* in Arabidopsis leads to water deficit tolerance. Under normal growth conditions, the *AtGolS2*-overexpressing lines showed both increased *AtGolS2* gene expression and Gol and Raf accumulation compared to the empty vector controls. When normally-grown three-week-old plants were exposed to soil drought stress by withholding water for two weeks and subsequently rehydrated for 5 d, the *AtGolS2*-overexpressing lines survived, but none of the control plants did, indirectly suggesting important roles of Gol and Raf in water deficit stress tolerance. GolS activities, *AtGolS2* expression and water-soluble carbohydrate concentrations during water deficit stress were not measured in that study.

A more fundamental research strategy would involve using a reverse genetic approach, exploiting a Raf-deficient Arabidopsis loss-of-function mutant. In context, as described in Chapter 3 of this work, this involved the identification of an *AtGolS2* homozygous T-DNA insertion mutant and a comparative analysis to wild type plants under water deficit conditions to determine if the absence of Raf leads to water deficit hypersensitivity. Only then can conclusions be drawn about the functional significance of stress-induced Raf in Arabidopsis.

### 1.7.2.2 The role of RFOs in cold acclimation and frost tolerance

The functional role of Raf in cold acclimation and frost tolerance is at present a matter of contradiction. A comparison of two Arabidopsis accessions with differing freezing tolerance (Col-0 and C24) demonstrated that Raf accumulated to a larger extent in the more tolerant accession (Col-0) in both non-acclimated and low temperature-acclimated (4°C) states (Klotke et al., 2004), suggesting a functional role for Raf in frost tolerance. Furthermore, transgenic petunia plants where an α-Gal was silenced contained higher levels of Raf and showed greater freezing tolerance in comparison to wild type plants (Pennycooke et al., 2003).

However, a comparative analysis of freezing tolerance in Arabidopsis T-DNA loss-of-function mutants for a putative RafS (*atrafs*) which are unable to accumulate Raf during low temperature-acclimation, and transgenic Arabidopsis plants constitutively overexpressing a *GolS* from cucumber and hyper-accumulating Raf showed no difference in freezing tolerance in either the non-acclimated or low temperature-acclimated (4°C) states (Zuther et al., 2004). These findings are astonishing as Raf has long been considered important in cold acclimation albeit from correlative observations that Raf increases in response to cold acclimation.

A possible explanation for the contradictions between petunia and Arabidopsis may be because the α-Gal which was silenced in petunia was an isoform with an acidic pH optimum, which may have a variety of substrates besides Raf, *in vivo*, and thus the freezing tolerance observed was due to an unknown pleiotropic effect. Alternatively, petunia accumulates RFOs up to Sta whilst Arabidopsis accumulates only Raf. Thus, the enhanced freezing tolerance in petunia may be due to Sta accumulation. Furthermore, the Arabidopsis study utilized plants that had been long term low temperature-acclimated (4°C, 14 d). We have recently demonstrated that excised leaves of *A. reptans* become more frost tolerant during long-term low temperature-acclimation (Peters and Keller, 2009) but that they accumulate less soluble carbohydrates (Inan-Haab and Keller, 2002). Nevertheless, we demonstrated that if RFO accumulation in this species is uncoupled from low temperature-acclimation then the frost tolerance of non-acclimated excised leaves positively correlates to RFO concentrations. We thus believe that long-term low temperature-acclimation may impart additive frost-protection, particularly given that a plethora of regulons are known to be induced in plants during low temperature-acclimation (Chinnusamy et al., 2007).

Since Arabidopsis only accumulates Raf, in vegetative tissues, during abiotic stress, a potential experimental approach to unequivocally answer the question of the role of Raf during cold acclimation would involve the over-expression of an alkaline α-Gal with a substrate specificity for Raf. Such an α-Gal has been functionally identified in *Cucumis melo* fruit (Gao and Schaffer, 1999). Expression of these isoforms in Arabidopsis should then, in theory, result in transgenic plants that are unable to accumulate Raf during stress but still retain other stress-inducible pathways. We describe such an approach in Chapter 4 of this work.

### 1.8 The dichotomy of proline (Pro) accumulation during abiotic stress

Among the known compatible solutes, Pro is perhaps the most widely distributed. The stress-induced accumulation of Pro has been noted to occur not only in plants but also in many bacteria, marine invertebrates, protozoa and algae (McCue and Hanson, 1990).

The accumulation of free Pro in plants, in response to abiotic stress, is so common that many researchers have inevitably studied its protective effects. However, as in the case with the RFOs, the functional role of Pro in plants during stress events is a matter of controversy (Verbruggen and Hermans, 2008). In unstressed situations, Pro accounts for about 5 % of the total free amino acid pool in plants. During various stress situations, large increases in free Pro of up to 80 % of the free amino acid pool occur (Matysik et al., 2002). As a compatible solute, Pro is thought to have multifunctional effects against osmotic stress and has been suggested to be a typical osmoprotectant, free radical scavenger and macromolecule protector. The accumulation of Pro in stressed plants is tightly controlled by the activation of its biosynthetic enzymes and the suppression of its hydrolytic enzymes (Nakashima et al., 1998).

Pro biosynthesis in plants is thought to occur via two biochemical pathways that share the intermediate glutamic-γ-semialdehyde (GSA, Fig 1.5). The first of these utilises glutamate as a substrate where, in two steps, the enzymes P5CS and P5CR convert glutamic acid into L-Pro. The second pathway utilises L-ornithine (Orn), where the enzyme orn δ-amino transferase (δ-OAT) catalyses the conversion of L-Orn to GSA. In higher plants, the accumulation of Pro during abiotic stress is linked predominantly to the glutamate biosynthesis pathway (Verma, 1999).

The *in planta* accumulation of Pro is a physiological dichotomy in higher plants as it has been reported that exogenous supply of Pro is toxic to *Nicotiana silvestris* cell suspension cultures and Arabidopsis plants (Bonner et al., 1996; Hellmann et al., 2000). *In planta* evidence where a Pro dehydrogenase enzyme was silenced in Arabidopsis (ProDH, the first committed enzyme in Pro catabolism), demonstrated that silenced lines were hyper-sensitive to exogenous Pro (Mani et al., 2002).

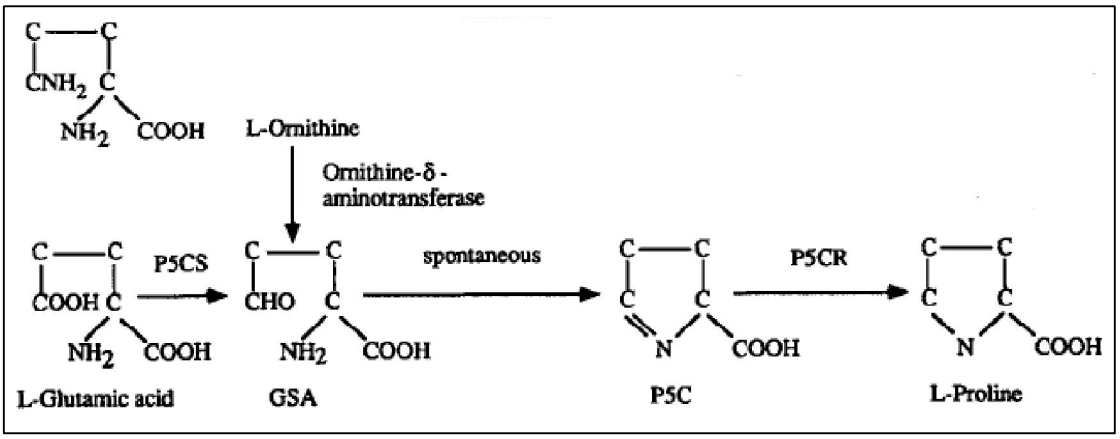


**Figure 1.5**: Schematic of the glutamate and ornithine proline biosynthetic pathway in plants. *P5CS*, ∆1-pyrroline-5-carboxylate synthase; *P5CR*, ∆1-pyrroline-5-carboxylate reductase. Adapted from Delauney and Verma (1993).

It was further reported that, in an Arabidopsis T-DNA loss-of-function mutant for *AtProDH*, exogenous Pro application led to high concentration increases of free Pro in the leaves in a dose dependent manner. Wild type plants did not show this, suggesting that the *AtProDH* gene is the master regulator of Pro catabolism in Arabidopsis and also demonstrating that it is free Pro itself and not catabolic intermediates, that cause the toxicity effects (Nanjo et al., 2003). However, it has also been reported that *in vitro*, P5CS exhibits feedback inhibition at Pro concentrations of about 10 mM, yet Pro itself may frequently occur in the leaves of plants exposed to water deficit at concentrations up to about 150 mM (Delauney and Verma, 1993). Collectively, such data alludes to an *in vivo* process that would appear much more complex than simple correlations between stress-induced Pro accumulation and plant performance. Indeed, the functional identification of Pro transporters in plants (Rentsch et al., 1996; Schwacke et al., 1999; Ueda et al., 2001; Rentsch et al., 2007) and the presence of Pro in the phloem of plants undergoing water deficit (Hocking, 1980; Girousse et al., 1996; Lee et al., 2009), suggest that Pro may exert a systemic protective effect rather than one localised to the cells in which it is produced.

Despite a lack of consensus as to how Pro may exert an *in vivo* functional effect during abiotic stress, it remains a metabolite that is extremely well characterised particularly in Arabidopsis, where it has been reported to increase during water deficit (Verslues and Bray, 2006), high salinity (Werner and Finkelstein, 1995; Liu and Zhu, 1997) and cold acclimation (Xin and Browse, 1998; Zuther et al., 2004; Fatma Kaplan, 2007). Changes in Pro concentration may thus be used as a positive marker of abiotic stress in Arabidopsis and may be particularly useful in reverse genetic studies using T-DNA loss-of-function mutants that show hyper-sensitive stress phenotypes.

### 1.9 Carbohydrates regulate gene expression in plants during development and abiotic stress

All organisms are obligately required to sense, and respond to, changes in the availability of metabolites. Given that life is defined by carbon-based organisms, metabolites such as carbohydrates are of paramount importance. Extensively studied responses to metabolite availability may be found in microorganisms, particularly bacteria (Görke and Stülke, 2008) and yeast (Gancedo, 1998; Carlson, 1999). Here, the phenomenon of catabolite repression sees very specific sets of genes in these organisms being regulated in the presence of Glc. Such observations directly link sugars to gene expression in these systems.

Research in plants has revealed that the carbohydrate status is responsible not only for gene expression but also repression (Stitt et al., 1991; Krapp et al., 1993; Sheen, 1994; Koch, 1996). Examples of carbohydrate-mediated gene regulation in plants have been found at the transcriptional, RNA processing and post-translational levels. Although this field remains in its infancy in plants, convincing data have been obtained from Arabidopsis, suggesting that plants contain multiple sugar sensing/response pathways and that some of these pathways share components with those from other organisms.

One of the earliest lines of evidence was obtained by the identification of two distinct hexokinase genes in Arabidopsis (*AtHXK1* and -*2*) that were able to functionally complement a Fru growth defect of a yeast hexokinase null mutant (hxk1$\Delta$hxk2$\Delta$) (Jang et al., 1997). Further characterisation of transgenic plants overexpressing these genes revealed distinct alterations in sugar responses in seedling development and gene expression, consistent with a functional role for the AtHXKs in sugar

signaling (Jang and Sheen, 1994). Further evidence for HXKs being *bona fide* sugar sensors in plants come from experiments conducted using Glc analogues. Exogenously feeding plants with 2-deoxglucose (phosphorylated by HXK but not further metabolised) causes alterations in the expression levels of sugar-related genes that are comparable to alterations observed when plants are supplied with Glc, suggesting that sugar signaling pathways do not overlap with downstream Glc metabolic pathways and furthermore placing the Arabidopsis HXKs as *bona fide* Glc sensors (Jang and Sheen, 1994).

The complexities of sugar signaling and gene expression networks in plants have been revealed using reverse genetic studies, particularly analysis of glucose insensitive (*gin*) mutants. One of these mutants, *gin2*, has firmly placed HXKs as central players in sugar dependent gene expression, integrating nutrient, light and hormone signaling pathways. When wild type and *gin2* mutants were grown under varying light conditions such that they accumulated different endogenous Glc concentrations, both plants were phenotypically similar when grown under low light intensities (Moore et al., 2003). However, at higher light intensities, *gin2* plants were significantly smaller and had dark green leaves. This observation led to the conclusion that AtHXK is somehow intricately involved in promoting growth, with the authors suggesting that the phenotype of *gin2* plants (delayed senescence and reduced fertility) has parallels to increased longevity observed in animal models in response to caloric restriction (Moore et al., 2003). Besides the use of gin mutants, a vast collection of other sugar-insensitive and -oversensitive mutants have been studied which include Suc-insentive growth (*sig*), Glc-super sensitive (*gss*), Suc-super sensitive (*sss*) (Pego et al., 2000), and Glc-oversensitive (*glo*) (Rolland et al., 2002). In the context of this thesis, findings from those studies will not be discussed and the reader is referred to the relevant references and current reviews (Baena-González and Sheen, 2008; Ramon et al., 2009).

An important point to note is that, to date, no conserved *cis*-acting elements have been reported in the promoters of sugar-regulated plant genes. However, it has been reported that, in *Zea mays*, a subset of sugar-regulated genes share conserved promoter motifs (Kim and Guiltinan, 1999). Whether these motifs represent *cis* elements that serve as recognition factors for the co-ordinate binding of transcription factor/s, remain speculative. Many questions still remain to be answered; (i) Does HXK sense glucose in a linear concentration dependent manner or does it act as a flux sensor? (ii) How do the down stream mechanism/s for gene

regulation work? (iii) Are signaling and gene expression pathways modulated in the plant cell to compartment specific loci? Significant advances in Arabidopsis resources, such as homozygous T-DNA loss-of-function mutant collections will undoubtedly expedite the elucidation of these pathways, but at present it is clear that the dual function of sugars as nutrients and signaling molecules complicates dissecting their role/s in the signal transduction pathways leading to differential gene expression.

A subset of stress-responsive genes have been demonstrated to be transcriptionally upregulated by Glc in Arabidopsis, suggesting that sugars may play a role in gene regulation during environmental stress responses in plants (Price et al., 2004). Furthermore, it has been reported in Arabidopsis, that under low temperature, water deficit and high salinity a total of 31 genes corresponding to enzymes involved in carbohydrate metabolism show differential stress-responsive regulation (Seki et al., 2002).

Simple sugars such as Glc and Suc are known to be efficient modulators of gene expression in plants (Koch et al., 1992; Ciereszko and Kleczkowski, 2002; Rolland and Sheen, 2005). Increases in soluble acid invertase activity have been observed in maize leaves subjected to water deficit (acid invertases hydrolyse Suc and occur in soluble form in the vacuole and in insoluble or soluble form in the cell wall)(Zinselmeier et al., 1999; Trouverie et al., 2003). Similar observations have been made in the leaves of mature bean (Castrillo, 1992) and pigeon pea (Keller and Ludlow, 1993). In all cases, the water deficit-induced increases in acid invertase activity correlated with increased hexose sugar concentrations. Another important enzyme in carbohydrate metabolism in plants is sucrose-phosphate synthase (SPS), a central player in controlling sucrose biosynthesis in higher plants (Huber and Huber, 1996; Li et al., 2003; Pan et al., 2009), catalysing the formation of Suc-6P from Fru-6P and UDP-Glc. Strong water deficit-induced decreases in SPS activity have been reported in the leaves of *Z. mays* (Pelleschi et al., 1997; Foyer et al., 1998), mature bean (Castrillo, 1992), Arabidopsis (Fresneau et al., 2007), and the resurrection plant *Craterostigma plantagineum* (Ingram et al., 1997). In all cases, Suc increases were correlated with a decrease in SPS activity. The complexities of these regulation pathways are again revealed in plants, as the genes encoding both soluble acid invertase and SPS are themselves subject to sugar regulation (Ehness et al., 1997; Pavlinova et al., 2002).

## 1.10 Plant models

### 1.10.1 *Arabidopsis thaliana*

*Arabidopsis thaliana* is a member of the Brassicaceae that has, over the last 15 years, become the model in plant biology research. Having been the first plant and only the third multi-cellular organism to have its genome fully sequenced (Bevan and Walsh, 2005) has led to the development of extensive functional genomics tools for researchers (Alonso et al., 2003).

Arabidopsis is a small plant with simple growth requirements that make it easy to grow under laboratory conditions. The plant has a short life cycle of about 8 weeks, is self fertilizing and produces thousands of seeds from a single parent, thereby facilitating the rapid production of many progeny from mutants or transgenic plants (Somerville and Koornneef, 2002). Arabidopsis is also easily transformed by exposing flowers to solutions of bacteria (*Agrobacterium* strains) harbouring a plasmid containing a gene of interest and then selecting for the presence of the transgene in the succeeding generation (Clough and Bent, 1998). A vast collection of more or less well-characterized mutations and transgenic plants is publicly available, in which many aspects of plant growth and development have been disrupted.

### 1.10.2 Is Arabidopsis a suitable model for studying abiotic stress tolerance?

Most contemporary molecular studies on stress tolerance use Arabidopsis. It is however important to iterate that this plant is a typical glycophyte (unable to tolerate moderate water loss or salinity exposures, Vinocur and Altman, 2005). It is moderately frost tolerant with the Columbia ecotype (Col-0) showing tolerance to temperature as low as -4.5°C in a non cold-acclimated state (Hannah et al., 2006). Intuitively, one would then conclude that, whilst Arabidopsis is a suitable model for the study of cold acclimation and frost tolerance, it is not suitable for studying osmotic or salt stress responses. The absence of genome sequences for typical xerophytes or halophytes makes them undesirable for intensive molecular and physiological studies.

A comparative analysis between the salt stress-responses of Arabidopsis and its salt-tolerant relative *Thellungiella halophila* suggested that the genes responsible for tolerance are the same as those found salt stress-induced in Arabidopsis. The difference in salt tolerance between these plants is thought to be that many of the

salt stress-induced genes in Arabidopsis are constitutively expressed in *T. halophila* (Taji et al., 2004).

Similarly, CBF/DREB-like proteins containing the signature features of Arabidopsis CBF/DREBs have been reported to occur in *Brassica napus* (Jaglo et al., 2001), barley (Choi et al., 2002), tomato (Jaglo et al., 2001) and rice (Dubouzet et al., 2003). Furthermore, the genes for putative CBF/DREB orthologs are transcriptionally upregulated during cold acclimation of *B. napus*, barley, tomato and rice. It is thus evident that whilst Arabidopsis is a glycophyte with respect to water deficit and high salinity, the genetic mechanisms involved in the regulation and expression of stress-induced genes appear to be conserved across many different plant species. Thus, Arabidopsis itself is a suitable plant model to understand the regulation of genetic pathways during abiotic stress.

### 1.10.3 *Ajuga reptans*

*A. reptans* is a frost hardy, evergreen Lamiaceae (labiate, Fig. 1.6) that has been used in our laboratory as a model to study the physiology of RFOs for over two decades.

This long standing research interest in the RFO metabolism of this plant has led to the discoveries that (i) RFOs mainly are the principal carbon translocates and carbon stores (Bachmann et al., 1994), (ii) RFOs are stored as higher oligomers in vacuoles of the leaf mesophyll and in the roots (Bachmann and Keller, 1995) and (iii) higher RFO oligomers are synthesised Gol-independently by GGT (Bachmann et al., 1994; Haab and Keller, 2002; Tapernoux-Lüthi et al., 2004) which is targeted to the vacuole via a novel sorting determinant (Tapernoux-Lüthi et al., 2007).

We have also long speculated that the frost tolerant nature of *A. reptans* is associated with the high concentrations of RFOs it contains during autumn and winter (up to 200 mg $g^{-1}$ FW in leaves, Bachmann *et al*., 1994), but have thus far never undertaken to quantify frost tolerance with respect to varying RFO concentration and chain length.

**Figure 1.6**: Naturally growing *A. reptans* plants in flower. Source www.upload.wikimedia.org

**Aims of this work**

We report in this work a multipronged strategy to further investigate the role/s of RFOs in abiotic stress tolerance. To this end, we describe (i) the development of an excised leaf system of *A. reptans* that uncouples RFO accumulation from cold acclimation and its use to examine the effect of RFO concentration in frost tolerance, (ii) Arabidopsis T-DNA loss-of-function mutants for the water deficit-induced At*GolS1* and -*2* genes, analysing their performance under water deficit and (iii) an Arabidopsis alkaline $\alpha$-Gal (*ATSIP2*) with a substrate specificity for Raf, that we functionally identified in Sf9 insect cells, and used as a tool to manipulate *in vivo* stress-induced Raf concentrations by constitutive overexpression in Arabidopsis.

We demonstrate that in *A. reptans* excised leaves frost tolerance positively correlates to increases in RFO concentrations in the absence of cold acclimation. Furthermore, the Arabidopsis *atgols2* loss-of-function mutant exhibits a hyper-sensitive to water deficit phenotype and accumulates less Raf, Suc and Pro suggesting that Gol may have additional effects on stress-induced signalling and compatible solute

accumulation. We also report on the functional identification of *ATSIP2*, by heterologous expression in Sf9 insect cells, showing it to be a *bona fide* alkaline $\alpha$-Gal with a substrate preference for Raf. We have overexpressed *ATSIP2* in Arabidopsis under the control of a constitutive and a water deficit-inducible promoter, as part of a strategy to generate transgenic plants which retain a functional stress-inducible RFO biosynthetic pathway but fail to accumulate Raf.

# Chapter II: Uncoupling RFO accumulation from low temperature-acclimation demonstrates that RFOs improve frost tolerance in *Ajuga reptans* L.

## 2.1 INTRODUCTION

*A. reptans* exists naturally in two distinct physiological states relative to RFO pools. It has been reported that during spring and summer in Zürich (April-August), RFO concentrations in this plant are relatively low, attaining concentrations of 74 and 42 mg $g^{-1}$ FW in aerial parts and roots, respectively (Bachmann et al., 1994). In the transition from late summer to autumn (late August), dramatic mass increases in RFOs occur, attaining maximum concentrations of 200 and 110 mg $g^{-1}$ FW in aerial parts and roots, respectively. Presumably, this is primarily the storage function of the total RFO pool, as concentration decreases occur in aerial parts and roots throughout late winter to summer (February to June) suggesting a remobilisation of the storage RFO pool toward growth.

Our work on the RFO physiology of *A. reptans* has long pointed toward a second role of these oligosaccharides in frost tolerance. The highest RFO concentrations co-incide with the winter season when plants are exposed to the most extreme of low temperatures in Zürich (average day temperature of between 0 and -5°C, http://www.meteoschweiz.ch/). In whole, soil-grown plants, we are only able to induce the accumulation of RFOs, in the laboratory, through cold acclimation (10°C/3°C, 16h/8h). Typically, RFOs begin accumulating after 4-6 weeks of acclimation. Given that a number of other low temperature-induced protective pathways come into play during long term cold acclimation (Chinnusamy et al., 2007), dissecting a functional role for RFOs in the frost tolerance of *A. reptans* is challenging.

Our aim, was therefore, to employ a system that would effectively uncouple RFO accumulation from low temperature acclimation, thereby allowing us to analyse frost tolerance as a function of RFO concentration. To this end, it has been previously demonstrated that excised leaves of *A. reptans*, placed in water-filled test tubes, rapidly accumulate long chain RFOs and increase GGT activity (Inan-Haab and Keller, 2002; Tapernoux-Lüthi et al., 2004). Here we report on the refinement of the excised leaf system, describing its physiological validation and use as a tool to analyze concentration-dependent effects of RFOs on frost tolerance in *A. reptans*. We were able to demonstrate that excision does not affect the fundamental carbohydrate physiology of a leaf and that the frost tolerance of excised leaves was improved as RFO concentration increased, suggesting a critical role for these oligosaccarides in the natural frost tolerance of *A. reptans*.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Plant material

*A. reptans* plants were grown in a controlled environment chamber (12 h light, 30 µmol photons $m^{-2}$ $s^{-1}$, 22°C, 12 h dark, 60% RH) in 6.5 L of Luwasa® hydroculture medium (0.3% v/v, Interhydro AG, Switzerland) which was continuously aerated using a home aquarium air pump. Growth medium was replaced weekly. Plants were considered experimentation ready once the root systems had reached approximately 20 cm in length and leaf rosettes were abundant. *Nicotiana benthamiana* plants were propagated from seeds under greenhouse conditions. After 7 d, plants were transplanted, one to a pot, and transferred into the growth chamber described above. Plants were considered experimentation ready after a further 7 d of growth in the chamber.

### 2.2.2 Leaf sets

Mature source leaves of similar size were excised from rosettes with a scalpel and the ends of the petioles subsequently re-cut under water. Petioles were immersed in a test tube, filled with water, and capped with a perforated black plastic cap that allowed the leaf petiole through. Leaf sets (six leaves per set) were then placed in a test tube rack covered with black PVC sheeting, exposing only the leaf to light, and maintained in the controlled environment chamber described above. One leaf set was subsequently sampled every 6 d over a period of 30 d and processed for carbohydrate and enzyme extractions, as well as frost tolerance experiments. *N. benthamiana* leaves were treated in the same manner, except that leaf sets were sampled at 6, 9 and 12 d.

### 2.2.3 Water soluble carbohydrate (WSC) extraction

WSCs were extracted using an ethanol series as previously described (Peters et al., 2007) with modifications. Two leaf discs (6 mm ∅) were punched out of each leaf in a set, weighed, flash frozen in liquid $N_2$, and macerated, using a plastic pestle in a 1.5 mL Eppendorf tube. WSCs were extracted twice (per step) in a three step sequential process, using 100 µL 10 mg $g^{-1}$ FW of 80% EtOH, 50% EtOH and $dH_2O$. Extractions were conducted at 85°C for 10 min and the tubes centrifuged at 12000 g (5 min, 4°C). Supernatants were removed to a separate Eppendorf tube before the next extraction in the sequence. The supernatants of all extractions were pooled and concentrated in a vacuum concentrator centrifuge. Extracts were then re-suspended

in 100 μL of $dH_2O$. Aliquots (50 μL) were de-ionised and de-phenolised as described below and analysed by HPLC-PAD.

### 2.2.4 Plant crude extracts

Freshly harvested leaf material (200 mg) was ground in 500 μL of chilled extraction buffer A for alkaline enzyme extracts [50 mM Hepes/KOH pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 20 mM DTT, 0.1% (v/v) Triton X-100, 1 mM benzamidine, 1 mM PMSF, 50 mM Na ascorbate, 2% (w/v) PVP] or extraction buffer B for acidic extracts [50 mM trisodium citrate, 20 mM DTT, 5 mM $MgCl_2$, 2% PEG 6000 (w/v), 2% PVP K30 (w/v) pH 5.0]. Samples were transferred into 2 mL Eppendorf tubes and centrifuged at 12000 *g* (10 min, 4°C). A 150 μL aliquot of supernatant was desalted by gel filtration at 1400 g (2 min, 4°C) through 5 mL Sephadex G-25 columns (fine, final bed volume of 3 mL). Columns were pre-equilibrated with an alkaline assay buffer (50 mM Hepes/KOH pH 7.5, 2 mM $MnCl_2$, 10 mM DTT) or an acidic assay buffer (McIlvaine Buffer pH 5.0). Pre-equilibration was performed twice by centrifugation at 1400 g (2 min, 4°C) each with 2 mL of assay buffer. Aliquots (20 μL) of desalted extract were assayed for respective enzyme activities in a final volume of 40 μL assay buffer containing 100 mM Suc and 10 mM Gol for RafS, 50 mM Raf and 5 mM Gol for StaS, 100 mM Raf for GGT, and both acidic and alkaline α-Gals. All assays were conducted for 1 h at 30°C and stopped by flash freezing the tubes in liquid $N_2$, and subsequently boiling for 5 min. Samples were de-ionised, de-phenolised, and analysed by HPLC-PAD.

### 2.2.5 Desalting of extracts

Desalting of carbohydrate and enzyme assay samples to remove phenolics and ions was conducted by centrifuge-rinsing of the samples through pre-rinsed 1 mL Mobicol spin columns (MoBiTec, Göttingen, Germany) as previously described (Peters et al., 2007) with minor modifications. Aliquots of ethanol extracts (75 μL) were desalted and centrifuge-rinsed twice, each with 175 μL of $dH_2O$. Desalted samples were then concentrated in a vacuum concentrator centrifuge and re-suspended in 100 μL of $dH_2O$ prior to HPLC-PAD. All 40 μL of enzyme assay reactions were desalted and centrifuge-rinsed once with 100 μL of $dH_2O$.

### 2.2.6 HPLC-PAD analysis

Desalted carbohydrate extracts and enzyme assay reactions were analysed and quantified by HPLC-PAD as described (Peters et al., 2007). Briefly, two

chromatographic systems using either a $Ca^{2+}$/$Na^{+}$-moderated ion partitioning carbohydrate column (Benson BC-100, BC200 columns, 7.8 x 300 mm; Benson Polymeric, Reno, Nevada, USA), or an anion exchange CarboPac PA1 column (4 x 250 mm; Dionex, Sunnyvale, CA, USA) were used to separate carbohydrates. Quantification was done using the Chromeleon v6.4 software package, against a series of 5 nmol of standard sugars, the concentration of which corresponded to the linear response range of both chromatographic systems. In the absence of commercially available higher RFO oligomer standards, long chain RFOs (DP $>$ 5) were quantified against a verbascose (Ver; DP 5) standard and are presented as Ver equivalents.

### 2.2.7 Pulse-chase experiments

Six excised leaves were maintained in the controlled environment chamber described above for 6 d, then placed in a glass thin layer chromatography chamber. Two 1.5 mL Eppendorf tubes were scotch taped to the sidewalls. To introduce $^{14}CO_2$ into the system, $NaH^{14}CO_3$ (Hartmann Analytic, Braunschweig, Germany) was added to the Eppendorf tubes (0.925 MBq $g^{-1}$ FW, specific activity 2183 MBq $mmol^{-1}$). Lactic acid was added to each of the tubes and the chromatography tank sealed with a glass lid lined with silicone grease. Leaves were subjected to strong illumination (460 µmol photons $m^{-2}$ $s^{-1}$) for 15 min and chased with $^{12}CO_2$ for 2 h, on the benchtop, without additional illumination. Leaves were then processed either for carbohydrate extractions described above or phloem exudation.

### 2.2.8 Phloem exudations

Phloem exudations were based on the method previously described (King and Zeevaart, 1974; Bachmann et al., 1994), with modifications. After pulsing leaves with $^{14}CO_2$, petiole ends were re-cut under exudation buffer (5 mM EDTA, 5 mM $KHPO_4$ pH 7.0), and placed in an Eppendorf tube filled with exudation buffer. Exudations were allowed to proceed for 2-4 h in a sealed, humidified chromatography chamber. Subsequently, the samples were analysed for $^{14}C$-carbohydrates by separation on a BC-100 column coupled to a FLO-ONE Radio chromatography detector (Model A-525X, Packard, Zürich, Switzerland).

### 2.2.9 Frost tolerance experiments

Leaf sets were harvested at various post-excision times (0, 6, 12, 18 and 24 d) and subjected to freezing temperatures of -5, -10, -15 and -20°C, in a programmable freezing chamber (RUMED, Blanc-Labo S.A., Lonay, Switzerland). Prior to being

placed in the chamber, leaves were transferred into plastic bags containing a few ice chips and the end of the bags tied off loosely with string. For each freezing temperature, the chambers were programmed to decrease (from -1°C) by -1°C $h^{-1}$ and hold at the designated temperature for 3 h before increasing by +1°C $h^{-1}$ until the hold temperature of -1°C was reached. Subsequently, the plastic bags were placed on ice to thaw. Two leaf discs (6 mm ∅) were punched out of each leaf. Discs were placed immediately into the wells of a six-well microtiter plate and incubated at room temperature for 1 h with agitation. Each well contained 1.5 mL of de-ionised water. The conductivity was measured for each well using a conductivity meter (Model 712, Metrohm, Zofingen, Switzerland) and this represented the initial leakage ($L_i$). Leaf discs were then flash frozen in liquid $N_2$, quickly re-immersed in the same well they had originally been in, and incubated for 1 h as described above. Conductivity was measured for each well and this represented the total leakage ($L_t$). Electrolyte leakage was expressed as a relative percentage of total leakage [($L_i$ $L_t^{-1}$)*100]. The temperature at which 50% relative leakage occurred from these data was defined as the $EL_{50}$. For each series of inductions, $EL_{50}$ values were obtained by plotting non-linear (sigmoidal) regression curves through scatter plots of the relative leakage data obtained for each freezing temperature.

## 2.3 RESULTS

### 2.3.1 The leaves of *A. reptans* plants exhibit two distinct RFO states with differing frost tolerance

HPLC-PAD analysis of total leaf WSCs indicated that warm-grown plants (22°C) accumulated proportionally lower amounts of total RFOs with Sta (DP4) being the highest RFO oligomer detectable (Fig. 2.1A). In cold-grown plants (8°C/3°C, day/night), RFOs were proportionally higher and were detectable as higher oligomers up to about DP9 (Fig. 2.1B). When leaves from both of these plants were exposed to a freezing stress of -20°C, relative electrolyte leakage differed greatly. In the leaves of warm-grown plants, it was > 90%, whilst it was 6% in the leaves of cold-grown plants, comparable to the relative leakage of un-stressed, warm-grown control leaves (Fig. 2.1C and D, respectively).

### 2.3.2 Excised leaves produce mainly $^{14}$C-Suc as a photosynthate and export mainly $^{14}$C-Sta as a phloem translocate in $^{14}CO_2$ photsynthetic pulse-chase experiments

Photosynthetic pulse-chase experiments using $^{14}CO_2$ were conducted with excised leaves at 6, 12 and 24 d after excision (DAE). Radio-HPLC analyses of the leaf WSCs indicated that the major proportion of photosynthetically fixed carbon at all time points consistently occurred as $^{14}$C-Suc, along with $^{14}$C-Raf, $^{14}$C-Sta, $^{14}$C-Gol, $^{14}$C-Glc and $^{14}$C-Fru (Fig. 2.2A, representative, 12 DAE). Radio-HPLC analyses of the EDTA phloem exudates from these leaves indicated that the predominant $^{14}$C-WSC in the phloem sap was $^{14}$C-Sta (Fig. 2.2B, representative, 12 DAE).

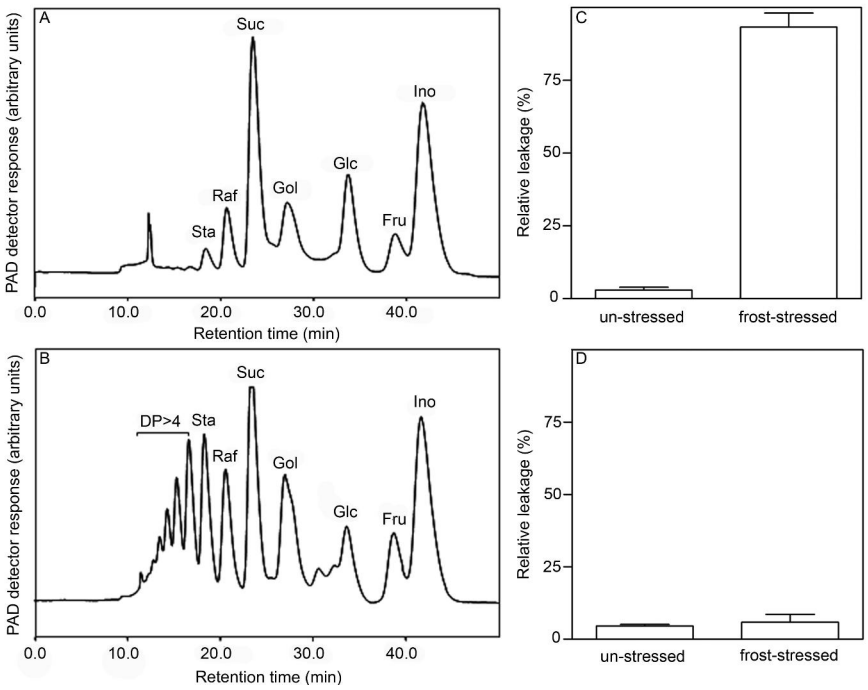


**Figure 2.1**: WSCs and frost tolerance of leaves from warm-grown (22°C, A,C) and cold-grown (8/3°C, day/night, B, D) *A. reptans* plants. Graphs A and B represent the WSC profiles whilst graphs C and D represent the relative electrolyte leakage from leaves, after exposure to a freezing stress of -20°C. Data points represent the mean ± SE of 6 replicates. Samples were analysed by HPLC using the BC 200 column.

### 2.3.3 Warm-incubated (22°C) excised leaves accumulate RFOs independent of cold acclimation

The changes in WSC concentrations were analysed in warm-incubated excised leaves at 6 d intervals over a period of 30 d. The total RFO concentration increased nearly 22-fold to 82 mg $g^{-1}$ FW (Fig. 2.3). Higher RFO oligomers (DP>4) accounted for the largest portion, showing a 74-fold increase from 0.6 to 57 mg $g^{-1}$ FW after 30 d. This correlated positively with an eight-fold increase in GGT activity from 5.6 to 46.4 nkat $g^{-1}$ FW. Sta increased 7.7-fold from 1.8 to 13.7 mg $g^{-1}$ FW. Raf increased seven-fold over the same period from 1.1 to 8 mg $g^{-1}$ FW. A clear positive correlation between Raf and RafS activity was not evident.

Suc concentration was nearly constant, showing a relatively minor 1.4-fold increase from 3.2 to 4.5 mg $g^{-1}$ FW (Fig. 2.3). Activities for RFO hydrolytic enzymes indicated that alkaline $\alpha$-Gal activity did not change over the 30 d period. However a pronounced increase in acidic $\alpha$-Gal activity occurred between 18 and 30 DAE (Fig. 3). Together with control leaves, we chose leaves at 12 and 24 DAE to

represent low, intermediate and high RFO states, respectively, to be subjected to frost tolerance experiments as described.

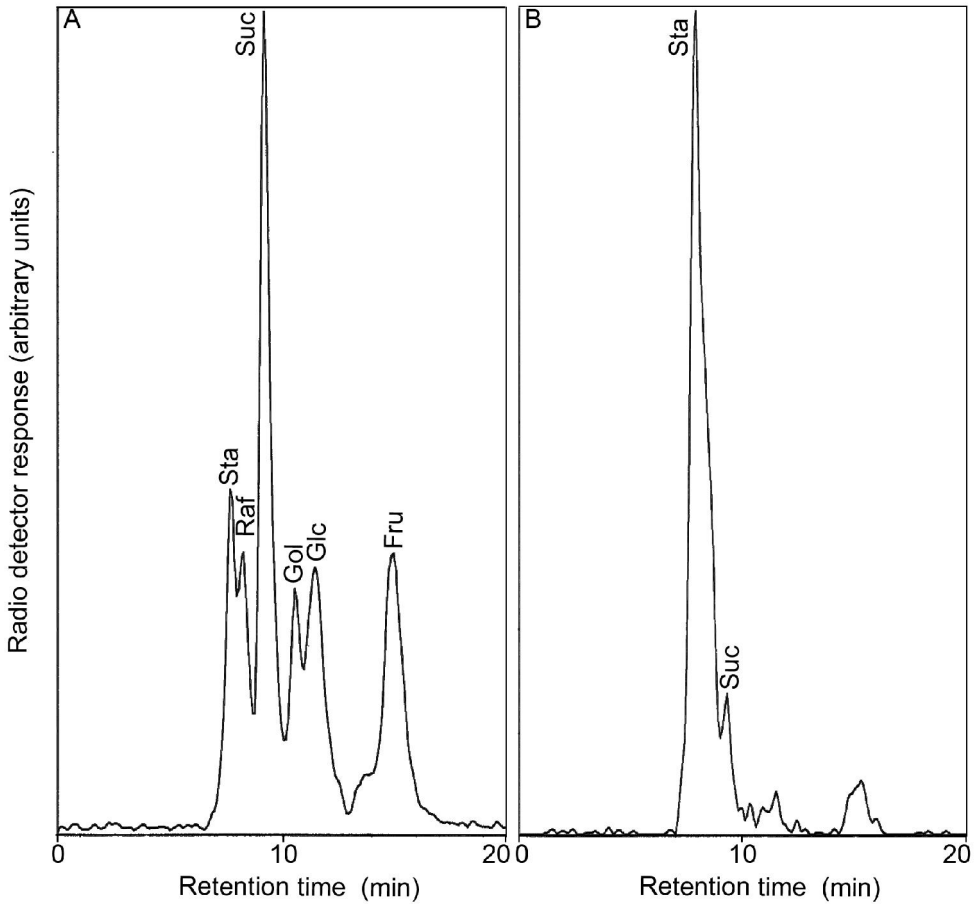


**Figure 2.2**: Representative radio-HPLC chromatogram of $^{14}$C-WSCs $^{14}CO_2$ photosynthetic pulse-chase experiments conducted on warm-incubated (22°C) *A. reptans* excised leaves (12 DAE), and analysed for carbohydrates (A) and phloem exudation (B). DAE, days after excision. Samples were analysed using the BC 100 column.

### 2.3.4 Electrolyte leakage and $EL_{50}$ are lower in leaves with a high RFO concentration

Excised leaves from 12 and 24 DAE were exposed to freezing temperatures of -5 to -20°C. Leaves at 24 DAE displayed the lowest relative electrolyte leakage over all temperatures tested (Fig. 2.4A). Similarly, leaves at 12 DAE showed an intermediate relative leakage between that of the control and 24 DAE. Temperatures of -15 and -20°C resulted in similar average relative leakage of 75% for controls, 60% for 12 DAE and 45% for 24 DAE, suggesting that -15°C is the upper limit of tolerance for leaves at these RFO states.

Excised leaves with a low RFO concentration (controls) had an $EL_{50}$ of about -10.5°C (Fig. 2.5A). As RFO concentrations in excised leaves increased, the $EL_{50}$ temperature decreased further with leaves showing an $EL_{50}$ of -16.0°C at 12 DAE and -24.5°C at 24 DAE (Fig. 5). The strongest correlation between $EL_{50}$ and RFO concentration was evident for higher RFO oligomers (> Sta). Excised leaves of *N. benthamiana* had no detectable RFOs over 12 DAE, when incubated under both cold and warm conditions. The frost tolerance of leaves incubated in the warm did not improve over this period, with leaves 12 DAE showing a slightly higher (75%) relative eletrolyte leakage than leaves 6 DAE, when challenged by freezing at -5°C (50%, Fig. 2.4B). Excised *N. benthamiana* leaves incubated in the cold showed a marked improvement in frost tolerance 6 DAE, compared to control leaves with relative electrolyte leakage of about 10% at -5°C but this tolerance did not improve over 12 DAE.

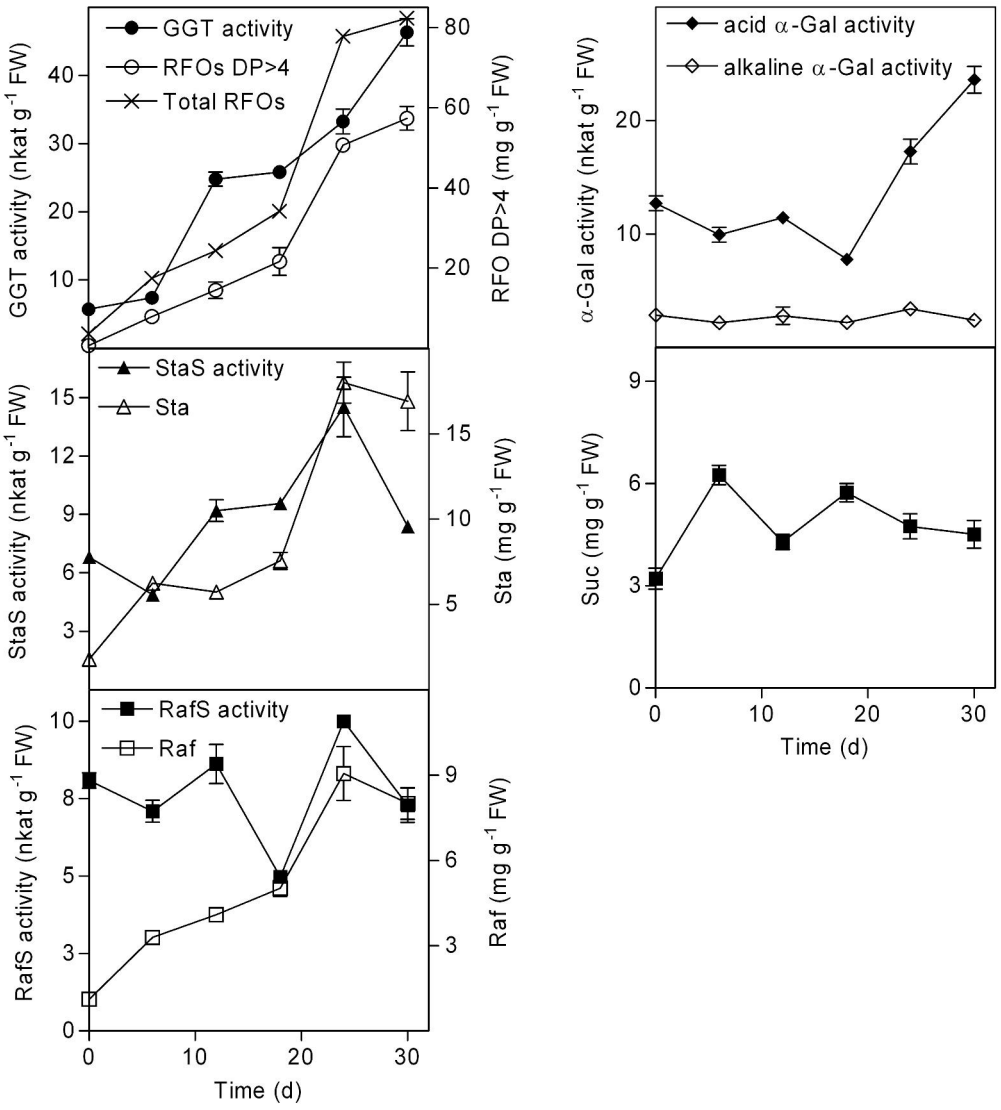


**Figure 2.3**: WSC and enzyme activity changes in warm-incubated (22°C) *A. reptans* excised leaves over a 30 d period. Datapoints represent the mean ± SE of 6 replicates. Enzyme assay samples were analysed by HPLC using the BC 100 column whilst WSC samples were analysed using both the PA1 and the BC100 columns.

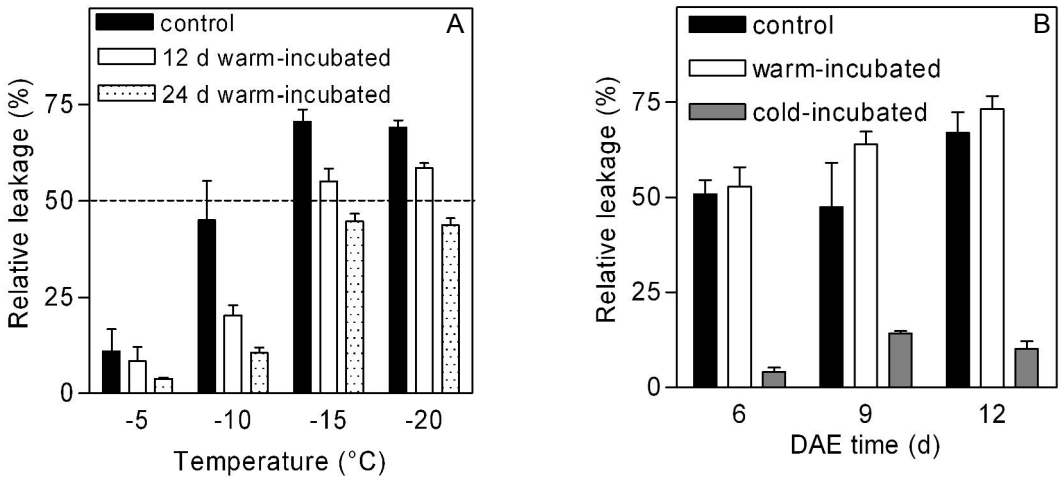


**Figure 2.4**: Relative electrolyte leakage of (A) warm-incubated (22°C) *A. reptans* excised leaves exposed to freezing temperatures of between -5 and -20°C, and (B) warm-incubated (22°C) and cold-incubated (8/3°C, day/night) *N. benthamiana* excised leaves exposed to -5°C. Control leaves were excised directly from warm-grown plants and subjected to freezing. Datapoints represent the mean ± SE of 6 replicates. DAE, days after excision.

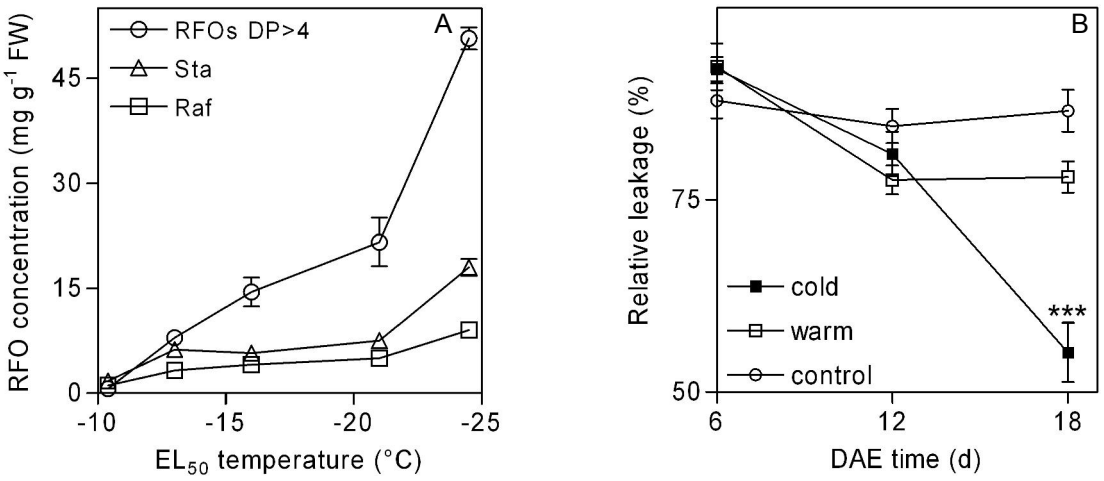


**Figure 2.5**: (A) $EL_{50}$ values plotted as a function of RFO concentration increases in warm-incubated (22°C) *A. reptans* excised leaves over a 30 d period. Data points represent the mean ± SE of 6 replicates at 6 d intervals, and (B) Relative electrolyte leakage of warm-incubated (22°C) and cold-incubated (8/3°C, day/night) *A. reptans* excised leaves exposed to a freezing stress of -20°C. Control leaves were excised directly from in warm-grown (22°C) plants and subjected to freezing at -20°C. Datapoints represent the mean ± SE of 6 replicates. Statistical significance of a two tailed t-test is represented by stars ($p<0.05$). DAE, days after excision.

## 2.4 DISCUSSION

### 2.4.1 *A. reptans* plants exist in two distinct physiological states that differ in frost tolerance

Our work on the RFO physiology of *A. reptans* has long pointed toward a second function of these oligosaccharides, i.e. in frost tolerance. The highest RFO concentrations co-incide with the winter season, when plants are exposed to the most extreme of low temperatures in Zürich (average day temperature of between 0 and -5°C, www.meteoschweiz.ch). Given that RFO mass increases up to at least Sta have been observed to occur in plants exposed to low temperature (Cunningham et al., 2003; Shahba et al., 2003; Klotke et al., 2004), we quantified the frost tolerance in leaves of cold-grown (8/3°C, day/night) and warm-grown (22°C) *A. reptans* plants.

The leaves of warm-grown plants accumulated proportionally lower amounts of RFOs, with Sta being the highest oligomer observed. The leaves of cold-grown plants, however, accumulated proportionally higher amounts of RFOs, with RFOs accumulating to higher oligomers (up to Suc-$[Gal]_7$, Fig. 2.1). Importantly, after a -20°C freezing stress, the leaves of cold-grown plants displayed a low relative electrolyte leakage that was comparable to un-stressed control leaves. Leaves of warm-grown plants showed high relative leakage values of > 90%, demonstrating that leaves from cold-grown plants are more frost tolerant. To further dissect a role for RFOs in this tolerance, we refined a previously described excised leaf system that would effectively uncouple RFO accumulation from cold acclimation (Inan-Haab and Keller, 2002).

### 2.4.2 Long term cold acclimation of excised leaves results in additive frost tolerance

Traditionally, we have induced RFO accumulation by exposing whole plants to a low temperature growth regime (8-10/3°C, day/night) to mimic the transition from late summer to autumn, when *A. reptans* naturally accumulates large amounts of RFOs (Bachmann et al., 1994). However, any attempt to analyse a functional role for RFOs in frost tolerance under this regime is met with the problem of the plethora of regulons induced during low temperature (Chinnusamy et al., 2007). Cold-incubated excised *A. reptans* leaves accumulate slightly less soluble sugars, in totality, than warm-incubated ones (Inan-Haab and Keller, 2002). However at 18 DAE, cold-

incubated excised leaves are more resistant to -20°C than warm-incubated ones (Fig. 2.5B). A traditional interpretation of such data would immediately discount a role for soluble sugars in frost tolerance. We however, maintain that under conditions of long term cold acclimation additive protective effects can mask the functional role/s of single components such as RFOs. To use an excised leaf system to examine the role of RFOs in the frost tolerance of *A. reptans*, we first examined the effects of excision on carbohydrate metabolism in leaves and compared these data to that of whole plants.

### 2.4.3 Excised leaves produce $^{14}$C-Suc and export $^{14}$C-Sta

The physiological validity of using an excised leaf system to uncouple RFO accumulation from cold acclimation in *A. reptans* was tested by inducing RFO accumulation over a period of 30 d under ambient growth conditions (22°C). Using classical photosynthetic $^{14}CO_2$ pulse-chase experiments, we demonstrated that at 6, 12 and 24 DAE single excised leaves operate within normal photosynthetic parameters, producing $^{14}$C-Suc as the major photosynthate, besides about 10% each of $^{14}$C-Gol, $^{14}$C-Raf, and $^{14}$C-Sta (Fig. 2.2A, representative, 12 DAE). This $^{14}$C-photosynthate pattern is quite similar to that obtained earlier using leaf discs and strips freshly isolated from *A. reptans* leaves (Bachmann and Keller, 1995). Furthermore, analysis of phloem exudates after pulsing excised leaves with $^{14}CO_2$ in the light indicated that the major WSC in the phloem is $^{14}$C-Sta (Fig. 2.2B, representative, 12 DAE), as we have previously reported using *A. reptans* whole plants (Bachmann et al., 1994). By removing a leaf from the plant the major physiological change that we propose to occur is the channeling of newly fixed carbon to the mesophyll vacuolar storage pool (Bachmann and Keller, 1995), without any measurable effect on (i) photosynthesis and (ii) the ability of the leaf to re-establish phloem transport through an incubation of the petiole excision zone in 5 mM EDTA. Obviously, the possibility of additional effects of leaf excision on frost tolerance cannot be totally excluded but might be minimal.

### 2.4.4 Excised leaves accumulate RFOs under warm growth conditions

As reported previously, RFOs increase in warm-incubated excised leaves (Inan-Haab and Keller, 2002). We have also measured GGT, RafS and StaS activities and, showing that GGT activity increases over 30 d correlate with the large increases in higher RFO oligomer concentrations (Fig. 2.3). Activity measurements for $\alpha$-Gals,

catalysing the first committed RFO degradation step, indicated that whilst alkaline α-Gal activity remained unchanged and low over a 30 d period, a very pronounced increase in acid α-Gal activity was observed after 18 DAE (Fig. 2.3). This could be correlated with the emergence of root buds at the excision zone of petioles, observed after 12 to 14 DAE. Assuming that this temporal window represents the point at which an excised leaf begins to reactivate its source activity, we postulate that this developmental switch triggers re-mobilisation of the RFOs in the mesophyll vacuoles and phloem export of carbon to fuel new root growth.

We have previously shown the phloem loading mechanism in *A. reptans* to be in accordance with the polymer trap model of symplastic loading (Turgeon and Gowan, 1990; Bachmann and Keller, 1995). This would imply that higher RFO oligomers in the vacuoles are re-mobilised by hydrolysis to Suc, which then diffuses into intermediary cells, to be immediately re-synthesised to Raf and Sta and exported via the phloem from the sieve elements. Unsurprisingly, Suc concentration remained relatively constant compared to RFOs, highlighting the fundamental physiology of *A. reptans* where post-photosynthetic carbohydrate metabolism is primed to favour RFO biosynthesis.

### 2.4.5 Frost tolerance in excised leaves correlates positively with RFO concentration

Most importantly, we have obtained a clear positive correlation between RFO concentrations and the frost tolerance of excised leaves, in the absence of cold acclimation. All control leaves had the lowest total RFO concentration (3.6 mg $g^{-1}$ FW) and were tolerant to -5°C, as evidenced by low relative electrolyte leakage (Fig. 2.4A). Total leaf RFO concentration increased nearly 22-fold in excised leaves in the warm, 24 DAE and leaves at 12 and 24 DAE were much more tolerant to freezing temperatures, up to -20°C, than control leaves. The total RFO increase correlated positively with a shift in the $EL_{50}$ value from about -10.5°C (control) to -24.5°C (Fig. 2.5A) showing for the first time quantitatively that, in *A. reptans* leaves, RFOs may improve frost tolerance irrespective of cold acclimation. The best correlation to the shift in $EL_{50}$ values was evident between concentration increases in higher RFO oligomers, which accounted for 65% of the total RFO increase. As a control, we also examined the effects of leaf excision on frost tolerance, using excised leaves of *N. benthamiana*. Our HPLC-PAD analysis indicated that no RFOs

occurred in either warm- or cold incubated excised leaves up to 12 DAE, in accordance with previous reports for tobacco (Haritatos et al., 1996). Importantly, excised *N. benthamiana* leaves incubated in the warm did not show any improvement in frost tolerance (Fig. 2.4B), despite a 3.5-fold Suc increase to about 418 mg $g^{-1}$ FW 12 DAE. A similar Suc increase (3.4-fold) was evident in cold-incubated excised *N. bentahmiana* leaves to about 384 mg $g^{-1}$ FW. However, frost tolerance was markedly improved in cold-incubated leaves 6 DAE, suggesting that other factors apart from Suc may be important in cold acclimation-induced frost protection of *N. benthamiana* excised leaves.

One of the *in vitro* experimentally demonstrated functions of the lowest RFO oligomer, Raf, is its ability to hydrogen bond to biomolecules more effectively than either Suc or trehalose, considered to be the most abundant stress protective sugars in nature (Gaffney et al., 1988). This direct hydrogen bonding between sugars and biomolecules has been demonstrated to be imperative, even in low concentrations, in the stabilization of proteins, membranes and whole cells under conditions of freezing and dehydration (Carpenter and Crowe, 1989; Prestrelski et al., 1993; Koster et al., 2000; Arakawa et al., 2001; Hincha et al., 2003; Hatanaka and Sugawara, 2008).

The protective efficacy of higher RFO oligomers in frost tolerance cannot be precluded on the basis of the compatible solute nature of these non-reducing sugars. Indeed, there is *in planta* evidence that together with Raf, Sta may play an important role in cold hardiness of *Loniceria caerula* L. shoot apices (Imanishi et al., 1998), alfalfa roots (Cunningham et al., 2003), as well as Chardonnay and Riesling grapevines (Hamman et al., 1996). From *in vitro* studies, it has been reported that commercially available RFO oligomers (up to Ver) offer increasing protection to artificial liposomes, during drying, with increasing chain length (Hincha et al., 2003). Similar *in vitro* observations were made for inulin type fructan oligomers ($\beta$2,1-fructosyl extensions of Suc, Suc-$[Fru]_n$) where lyo-protection was positively correlated with increasing chain length (Cacela and Hincha, 2006).

Our data has provided convincing *in planta* evidence that frost tolerance in *A. reptans* leaves may depend on RFO concentration since the excised leaf system uncouples RFO accumulation from cold acclimation. Furthermore, the best positive correlation to increases in $EL_{50}$ were evident for higher RFO oligomers (> Ver), suggesting that they may be important contributors to frost tolerance in this plant. Because these

higher RFO oligomers are synthesized and stored in the large central vacuole of mesophyll cells (Bachmann and Keller, 1995), the question arises if they may exert their protective role also outside the vacuole, e.g. in the plasma membrane. To do so, they would need to reach the plasma membrane, but no such mechanism has been described for RFOs nor has their putative apoplastic location been determined. The recently proposed model for vesicle-mediated transport of fructans from the vacuole to the apoplast to assist in stabilising the plasma membrane (Valluru et al., 2008) might also be applicable to RFOs and deserves further attention.

In conclusion, we have adapted a single excised leaf system for *A. reptans* that effectively uncouples RFO accumulation from low temperature. After physiologically validating that excision does not alter the fundamental carbohydrate metabolism of leaves, we used the system as a tool to demonstrate that frost tolerance in the leaves of *A. reptans* correlates positively with the concentration of accumulated RFOs, suggesting a protective role for these oligosaccharides in the natural frost tolerance of *A. reptans.* Present strategies to understand the role of higher RFO oligomers in frost tolerance are underway in our laboratory. A transformation system for *A. reptans* is being optimised and we envisage that excised leaves from transgenic plants, where GGT has been silenced, will provide key information to understanding the fundamental importance of these higher RFOs in frost tolerance. In addition we are pursuing an approach transforming Arabidopsis with GGT, since this plant does not accumulate RFO oligomers beyond Raf. We envisage that transgenic Arabidopsis over-expressing GGT will accumulate higher RFO oligomers and plan to evaluate the frost tolerance of these lines to ascertain if higher RFO oligomers are able to improve the frost tolerance of transgenic plants.

# Chapter III: An Arabidopsis T-DNA insertion mutant for *AtGolS2* is hypersensitive to water deficit, failing to accumulate Gol, Raf and Suc

## 3.1 INTRODUCTION

The use of Arabidopsis as a model to study the roles of RFOs in abiotic stress tolerance has a number of distinct advantages. The obvious first is that it is a model organism with a fully sequenced genome, coupled to a vast molecular toolbox which includes the availability of inventoried full length cDNAs and collections of loss-of-function mutants in which many aspects of plant growth and development are disrupted. Furthermore, in the context of RFO physiology, Sta is the highest detectable RFO oligomer in Arabidopsis seeds. Together with Raf, these two RFOs occur in high abundance only in mature seeds and are rapidly mobilized during the germination process. RFOs (Raf) are detectable in only trace amounts, in vegetative tissue of plants. However, upon exposure to a variety of abiotic stress very marked mass increases in both Gol and Raf has been reported to occur in leaves (Taji et al., 2002; Panikulangara et al., 2004; Nishizawa et al., 2008). Presumably, these increases are due to an elevated activity of RafS enzyme/s. However, most emphasis has rather been placed on GolS in relation to stress-induced Raf mass increases, perhaps because GolS is considered to be the rate limiting step in RFO biosynthesis (Karner et al., 2004; Kaplan et al., 2007).

Analysis of stress-induced GolS expression of the ten GolS genes in Arabidopsis has elucidated that a differential, stress-induced transcriptional up-regulation occurs (Taji et al., 2002; Nishizawa et al., 2008). *AtGolS1*, *-2*, *-3*, *-4* and *-8* are upregulated by oxidative stress (Nishizawa et al., 2008). *AtGolS1* and *-2* are upregulated by water deficit and high salinity, whilst AtGolS3 is upregulated by low temperature (Taji et al., 2002). *AtGolS1* has also been demonstrated to be transcriptionally upregulated by heat shock (Panikulangara et al. 2004). Thus, a model system that selectively accumulates one RFO oligomer (Raf) in response to abiotic stress and displays differential stress-induced gene expression to accumulate this Raf allows one to dissect the role of RFOs during stress, using a reverse genetic approach to analyse loss-of-function mutants for key RFO biosynthetic genes.

In a pioneering study, Taji et al. (2002) provided some molecular evidence that the constitutive overexpression of *AtGolS2* in Arabidopsis leads to water deficit tolerance. Under normal growth conditions, the *AtGolS2*-overexpressing lines showed both increased *AtGolS2* gene expression and Gol and Raf accumulation compared to the empty vector controls. When normally-grown three-week-old plants were exposed to soil drought stress by withholding water for two weeks and

subsequently rehydrated for 5 d, the *AtGolS2*-overexpressing lines survived, but none of the control plants did, indirectly suggesting important roles of Gol and Raf in water deficit stress tolerance. GolS activities, *AtGolS2* expression and water-soluble carbohydrate concentrations during water deficit stress were not measured in that study.

Our research methodology in the following chapter describes a strategy that exploited the availability of published work describing the differential up-regulation of GolS genes during abiotic stress (Taji et al., 2002). We report on the identification of homozygous T-DNA loss-of-function mutants for the *AtGolS1* and *-2* genes and their use to determine if an absence of Raf during exposure of the plants to water deficit.

## 3.2 MATERIALS AND METHODS

Unless otherwise stated, experiments were conducted at least twice; for each experiment, 21 individual replicates were used, with plants being divided into 3 pools of 7 plants.

### 3.2.1 Plant material and growth conditions

All experiments were conducted with the *Arabidopsis thaliana* (Col-0) genetic background. The *AtGolS2* mutant (Salk_075769) was obtained from the Salk Institute's T-DNA insertion mutant collection (Alonso et al., 2003). The mutant carries a T-DNA insertion in the promoter region of the gene. Homozygous mutant plants were identified using a triplex primer PCR. The wild type allele (~0.9 kB) was amplified using the primers: *GS2*f 5' CAACTTCTTTGGAACAAACAATG and *GS2*r 5'AATAGACCAAACGGGAAATGG, the mutant allele (~ 0.5 kb) was identified using the LBb1.3 T-DNA specific primer 5' GCGTGGACCGCTTGCTGCAACT and *GS2*r.

To generate *atgols1 atgols2* double mutants, we obtained a T-DNA insertion mutant recently described for *AtGolS1* (Nishizawa et al., 2008). Using the same strategy described above, homozygous plants were identified with the primers *GS1*f 5' AGAAAACATGGAGTCACACACGC and *GS1*r 5'TTCGAAACAAAAATTGAACCG. Homozygous *atgols1* plants were crossed with homozygous *atgols2* plants. Plants ($F_1$) heterozygous for T-DNA insertions in both *AtGolS1*and *-2* were identified by PCR analysis as described above and allowed to self-pollinate. Individual $F_2$ plants were screened for homozygous T-DNA insertion in both *AtGolS1* and *AtGolS2*. Progeny ($F_3$) were designated *atgols1 atgols2*, representing plants that carried T-DNA insertions in both *AtGolS1* and *-2*.

Following seed stratification (48 h, 4°C), plants were propagated on soil (Einheitserde, type ED73, Einheitserde-und Humuswerke Gebr. Patzer GmbH & Co. KG, Schopfheim, Switzerland) in a controlled environment chamber (8 h light, 120 μmol photons $m^{-2}$ $s^{-1}$, 22°C, 16 h dark, 60% relative humidity). Plants were transferred into new pots as described below after one week.

### 3.2.2 Water deficit

One week after sowing seeds, five plantlets were transplanted into pots (6x6x7 cm; Desch Plantpak, Waalwijk, Netherlands) containing 60 g of soil that had been sieved (0.5 cm mesh size) to remove large detritus and maintained in the controlled environment chamber described above. Six week old plants were challenged with water deficit by witholding water over a period of 10 d. Leaf tissue was sampled at appropriate time points (0, 3, 5, 6, 7, 8, 9 and 10 d). Leaf relative water content (RWC), leaf relative electrolyte leakage (REL) and GolS activity were determined immediately. Leaf material was also flash frozen in liquid $N_2$ and stored at -80°C for water-soluble carbohydrate analyses.

### 3.2.3 Leaf relative water content

Leaf RWC was determined at each sampling point as follows. Leaf pieces were excised and the initial weight (*Wi*) of each sample was recorded before immersing it in de-ionised water for 24 h. The weight at full turgor (*Wt*) was recorded and leaf samples subsequently dried at 55°C for 24 h, and the dry weight (*Wd*) was recorded. The *RWC* was calculated using the formula of (Barrs and Weatherley, 1962), *viz*. *RWC* = [(*Wi*–*Wd*)/*Wd*]/ [(*Wt*–*Wd*)/*Wd*]*100.

### 3.2.4 Leaf electrolyte leakage

Six leaf discs (7 mm ∅) were punched out of separate plants. Discs were placed immediately into the wells of a six-well microtiter plate and incubated at room temperature for 1 h with agitation. Each well contained 2.5 mL of de-ionised water. The conductivity was measured for each well using a conductivity meter (Model 712, Metrohm, Zofingen, Switzerland) and this represented the initial leakage (Li). Leaf discs were then flash frozen in liquid N2, quickly re-immersed in the original well and incubated for 1 h as described above. Conductivity was measured for each well and this represented the total leakage (Lt). Electrolyte leakage was expressed as a relative percentage of total leakage [(Li/Lt-1)*100].

### 3.2.5 Enzyme extractions and GolS activity assays

Freshly harvested leaf material (200 mg) was ground in 400 μL of chilled extraction buffer [50 mM HEPES/KOH pH 7.5, 5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 20 mM dithiothreitol (DTT), 0.1% (v/v) Triton X-100, 1 mM benzamidine, 1 mM phenylmethylsulphonyl fluoride (PMSF), 50 mM Na-ascorbate, 2% (w/v)

polyvinylpyrrolidone (PVP)]. Samples were transferred into 2 mL Eppendorf tubes and centrifuged at 12000 g (10 min, 4°C). A 150 μL aliquot of supernatant was desalted by gel filtration at 1400 g (2 min, 4°C) through 5 mL Sephadex G-25 columns (fine, final bed volume of 3 mL). Columns were pre-equilibrated with assay buffer (50 mM Hepes/KOH pH 7.5, 2 mM $MnCl_2$, 10 mM DTT). Pre-equilibration was performed twice by centrifugation at 1400 g (2 min, 4°C) each with 2 mL of assay buffer. Aliquots (20 μL) of desalted extract were assayed for GolS activity in a final volume of 40 μL assay buffer containing 5 mM UDP-Gal and 50 mM myo-inositol. Reactions were incubated at 30°C for 20 min and stopped by flash freezing the tubes in liquid $N_2$ and subsequently boiling for 5 min. Samples were de-ionised, de-phenolised, and analysed by HPLC-PAD as described below. GolS activity was expressed on a dry-weight basis by calculating the absolute tissue mass at each sampling time based on the RWC calculations described above.

### 3.2.6 RNA isolation and semi-quantitative PCR

Total RNA was extracted using the Plant RNeasy Mini kit (Qiagen AG, Hombrechtikon, Switzerland). The cDNA template for the semi quantitative PCR was obtained by reverse transcription of 1 µg total RNA with an oligo ($dT_{15}$) primer and M-MLV ($H^-$) reverse transcriptase (Promega AG, Dübendorf, Switzerland) according to the manufacturer's protocol. The semi quantitative PCR was carried out in a final volume of 50 µL containing 1 μL cDNA, 1.25 U GoTaq DNA polymerase (Promega), 1×PCR buffer, 0.5 mM of each dNTP, and 0.5 μmol of each primer. Reactions were conducted at a primer annealing temperature of 58°C for 22 cycles. The number of cycles chosen for the sqPCR was determined to occur in the linear range of the constitutively expressed *RPS16A* gene (At2g09990, encoding a 40s ribosomal protein). The *RPS16A* primer pair (*RPS16A*$_{fwd}$ 5' GGCGACTCAACCAGCTACTGA and *RPS16A*$_{rev}$ 5'CGGTAACTCTTCTGGTAACGA) amplified a 0.8 kB fragment of the cDNA.

The *AtP5CS* (At2g39800) primer pair (*AtP5CS*$_{fwd}$ 5' ATGGAGGAGCTAGATCGTTC and *AtP5CS*$_{rev}$ 5'TAGTAACATTTGCTTCAAGG) amplified a 1.0 kb fragment of the cDNA. The *AtGolS1* and *AtGolS* primer pairs (*AtGolS1*$_{fwd}$ 5'ATGGCTCCGGGGCTTA CTCA, *AtGolS2*$_{rev}$ 5'TCAAGCAGCGGACGGTGC, *AtGolS2*$_{fwd}$ 5'ATGGCACCTGAGA TCAATAC, *AtGolS2*$_{rev}$5' CTAAGCTGCAGATGGAGCTT) amplified the open reading frames (~1.0 kb) of both genes.

### 3.2.7 Water-soluble carbohydrate (WSC) extraction

WSCs were extracted from 15 mg of ground, freeze-dried leaf material representing leaf RWCs of 90, 80 and 45%, using an ethanol series as previously described (Peters et al., 2007; Peters and Keller, 2009), with modifications. Extractions were conducted twice (per step) in a two step sequential process, using 80% and 50% EtOH (v/v, 750 μL). The supernatants of all extractions were pooled and aliquots of extract (2 mL) were then concentrated on a vacuum concentrator centrifuge and re-suspended in 200 μL of de-ionised $H_2O$. Aliquots (100 μL) were then de-ionised and de-phenolised as previously described (Peters et al., 2007; Peters and Keller, 2009).

### 3.2.8 HPLC-PAD analysis

Desalted carbohydrate extracts and enzyme assay reactions were analysed and quantified by HPLC-PAD as previously described (Peters et al., 2007; Peters and Keller, 2009) using the BC100 chromatographic system.

## 3.3 Results

### 3.3.1 *AtGolS1* is transcriptionally upregulated in the leaves of *atgols2* plants during water deficit

Both *AtGolS1* and *-2* transcripts were not detected in the leaves of wild type (Col-0) Arabidopsis plants at full turgor as determined by semi quantitative PCR of first strand cDNA preparations (Fig. 3.1B). An increase in the transcripts of both these genes was evident in water deficit-stressed leaves of Col-0 plants. *AtGolS2* transcripts were not detected in the leaves of *atgols2* plants which carry a T-DNA insertion in the promoter of *AtGolS2* (Fig. 3.1A) nor in *atgols1 atgols2* plants at full turgor or under water deficit-stress. *AtGolS1* transcripts were not detected in *atgols1 atgols2* plants but increased in the leaves of water deficit stressed *atgols2* plants. As a positive control, we examined the effects of water deficit on *AtP5CS*, a gene believed to be the principle contributor to water deficit-induced proline mass increase in the leaves of Arabidopsis (Székely et al., 2008). Low transcript levels were evident in all plants under full turgor conditions. However, in leaves of water deficit-stressed plants , transcripts were much more abundant in Col-0, *atgols2* and *atgols1 atgols2* plants (Fig. 3.1B).

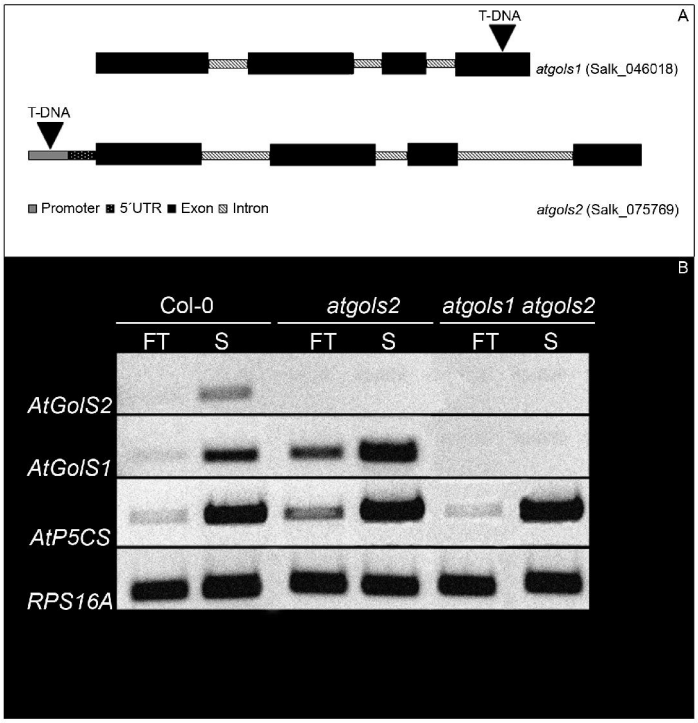


**Figure 3.1**: Genetic organisation of *atgols1* and *atgols2* T-DNA insertion mutants (A) and semi-quantitative reverse transcription (sqPCR) of gene expression in the leaves of unstressed (FT, full turgor) water stressed (S, stressed) Arabidopsis wild type plants and *atgols* T-DNA insertion mutants (B). *AtP5CS* (Δ1-pyrroline-5 carboxylate synthase, At2g39800) is a water deficit-induced proline biosynthetic gene and *RPS16A* (At2g0990) is a constitutively expressed 40s ribosomal gene. The *atgols1 atgols2* double T-DNA insertion mutant was created by standard crossing procedures outlined in the materials and methods.

### 3.3.2 Leaf water loss is more rapid in *atgols2* and *atgols1 atgols2* plants during water deficit

The leaf RWC was compared between Col-0, *atgols2* and *atgols1 atgols2* plants over a water-deficit period of 10 d. Plants displayed similar leaf RWCs of between 80-85% up to 6 d of water deficit. After 7 d, leaf RWC declined sharply in both *atgols2* and *atgols1 atgols2* plants to 53 and 60%, respectively (Fig. 3.2A). This rapid decline was further evident at 8 d of water deficit with leaf RWC reaching 32 and 36% in *atgols1* and *atgols1 atgols2* plants, respectively. Col-0 plants maintained a leaf RWC of about 80% until 7 d of water deficit. Thereafter, the leaf RWC declined to 63% (8 d) and 40 % (9 d; Fig. 3.2A). Leaf REL correlated negatively with the decline in RWC. Leaf REL increased slightly but significantly in *atgols2* and *atgols1 atgols2* plants between 7-8 d of water deficit. Leaf REL rose sharply at between 8-9 d of water deficit in these plants to about 60 and 50%, respectively. This coincided with a decline in leaf RWC to below 40% that under our experimental conditions represented a lethal event. Col-0 plants showed a similar REL pattern but the leaf RWC decline to below 40% occurred a full day later than in *atgols2* and *atgols1 atgols2* plants. Concomittant to the rapid decrease of leaf RWC in *atgols2* and *atgols1 atgols2* plants, they showed a visible loss of leaf turgor after 8 d of water deficit (Fig. 3.3), when the leaves of Col-0 plants remained fully turgid.

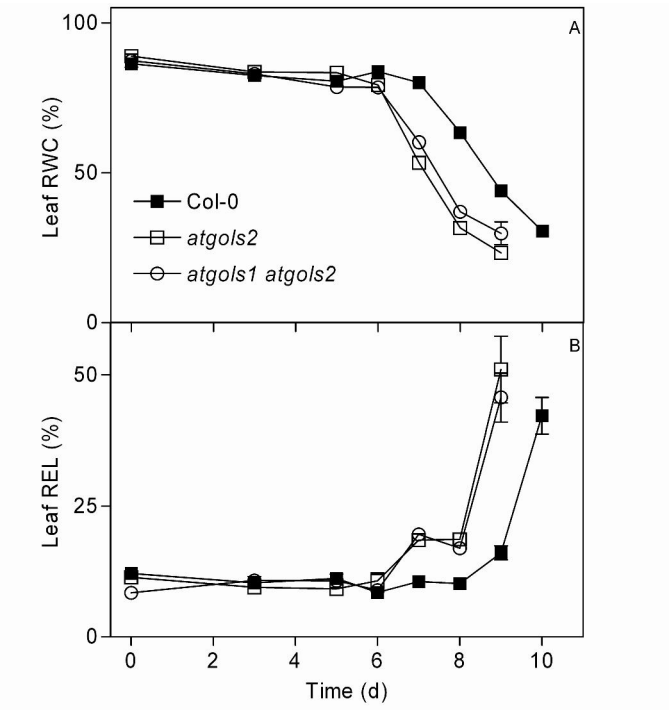


**Figure 3.2**: Changes in the leaf relative water content (RWC, A) and leaf relative electrolyte leakage (REL, B) of Arabidopsis wild type plants and a*tgols* T-DNA insertion mutants challenged with water deficit. Data points represent the means±SE of six replicates. (B). Datapoints for *atgols2* and *atgols1 atgols2* plants for 10 d water deficit are absent as this represented a lethal RWC threshold (40%) for these plants.

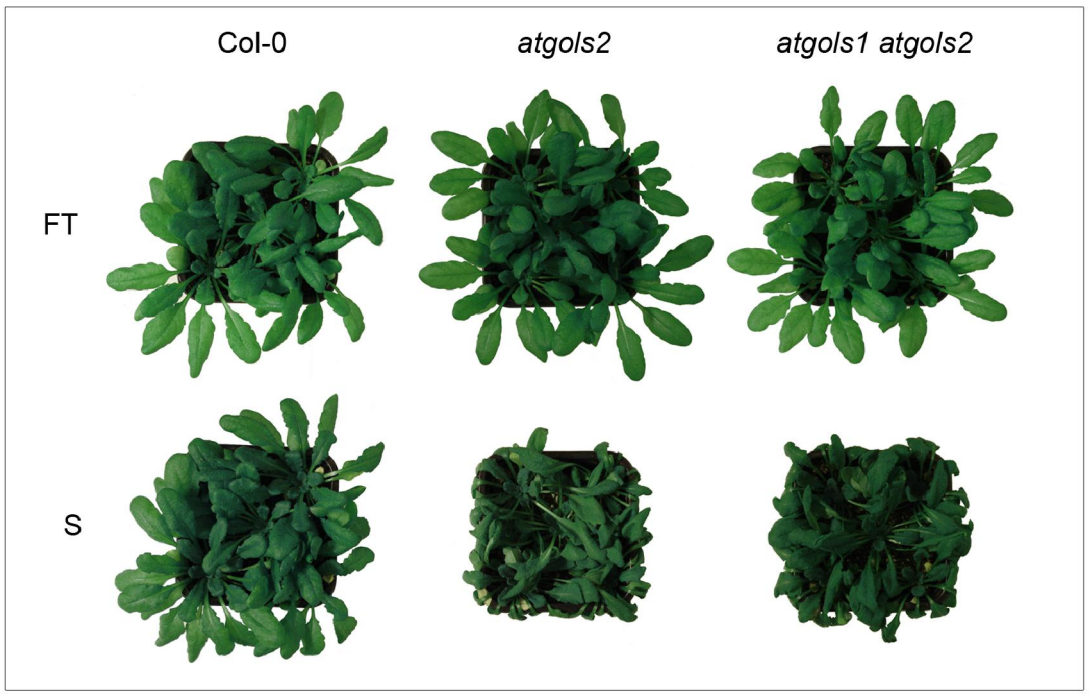


**Figure 3.3**: Phenotypic response of Arabidopsis wild type and *AtGolS* T-DNA insertion mutant plants challenged with water deficit. Stressed (S) plants represent a 7 d water deficit, when *atgols2* and *atgols1 atgols2* plants showed a a visible loss of leaf turgor and represents leaf relative water contents (RWC) of 53 and 60%, respectively. FT, Full turgor.

### 3.3.3 Gol, Raf and Suc fail to accumulate in water deficit-stressed leaves of *atgols2* and *atgols1 atgols2* plants

The GolS activity was measured in the leaves at full turgor and after water deficit stress (55-60% RWC). A 20-fold increase in GolS activity to 2.7 nkat $g^{-1}$ DW occurred in the leaves of water deficit-stressed Col-0 plants (Fig. 3.4). A 3-fold increase to 0.37 nkat $g^{-1}$ DW occurred in the leaves of water deficit-stressed *atgols2* plants. However, GolS activity remained unchanged in the leaves of water deficit-stressed *atgols1 atgols2* plants and was comparable to the activity in the leaves of all plants at full turgor (0.15-0.2 nkat $g^{-1}$ DW, Fig. 3.4). Concomittant to the lack of water deficit-induced GolS activity in the leaves of *atgols2* and *atgols1 atgols2* plants, Raf accumulation was severely attenuated in water deficit-stressed leaves of these plants showing only a 1.3-fold increase to 20 and 23 µg $g^{-1}$ DW, respectively (Fig. 3.5). Conversely, water deficit-stressed leaves of Col-0 plants had a Raf concentration of about 102 µg $g^{-1}$ DW, representing a 5.7-fold increase from leaves at full turgor. The two substrates for Raf biosynthesis, Suc and Gol, also showed differences in the mutant plants with Suc concentrations in stressed leaves of Col-0 plants showing a 20-fold increase to about 2000 µg $g^{-1}$ DW and Gol, showing a 2.7-fold increase to about 57 µg $g^{-1}$ DW, respectively. In *atgols2* and *atgols1 atgols2* plants, Suc

concentrations increased only 2.7 and 3.1-fold to about 990 and 718 µg $g^{-1}$ DW, respectively, in water deficit-stressed leaves. GoI concentration increased marginally (1.7-fold) to about 41 µg $g^{-1}$ DW in stressed leaves of *atgols2* plants, but did not change in stressed leaves of *atgols1 atgols2* plants. Ino concentrations were 2.5 and 1.5-fold higher in stressed leaves of *atgols2* and *atgols1 atgols2* plants (about 90 and 80 µg $g^{-1}$ DW, respectively; Fig. 3.5).

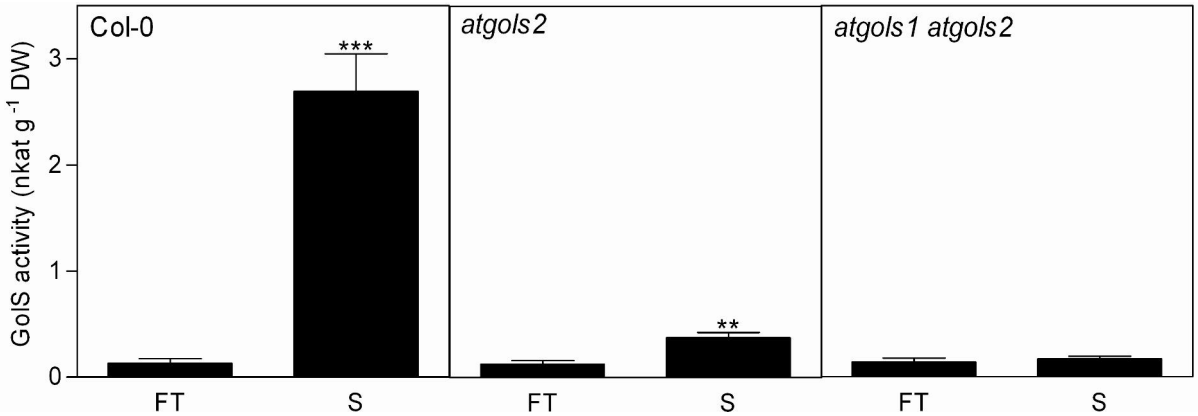


**Figure 3.4**: Changes in GolS activity in the leaves of Arabidopsis wild type plant and *AtGolS* T-DNA insertion mutants challenged with water deficit. Stressed (S) plants represent a 7 d water deficit, when *atgols2* and *atgols1 atgols2* plants showed a loss of leaf turgor and represents leaf relative water contents (RWC) of 53 and 60%, respectively. Col-0 plants (S) represent a 9 d water deficit when these plants showed visible loss of leaf turgor and represents a leaf RWC of 63%. Data points represent the means±SE of six replicates. Statistical significance of a two-tailed t-test is represented by stars ($p < 0.003$, Col-0 and $p < 0.0069$, *atgols2*). FT, Full turgor.

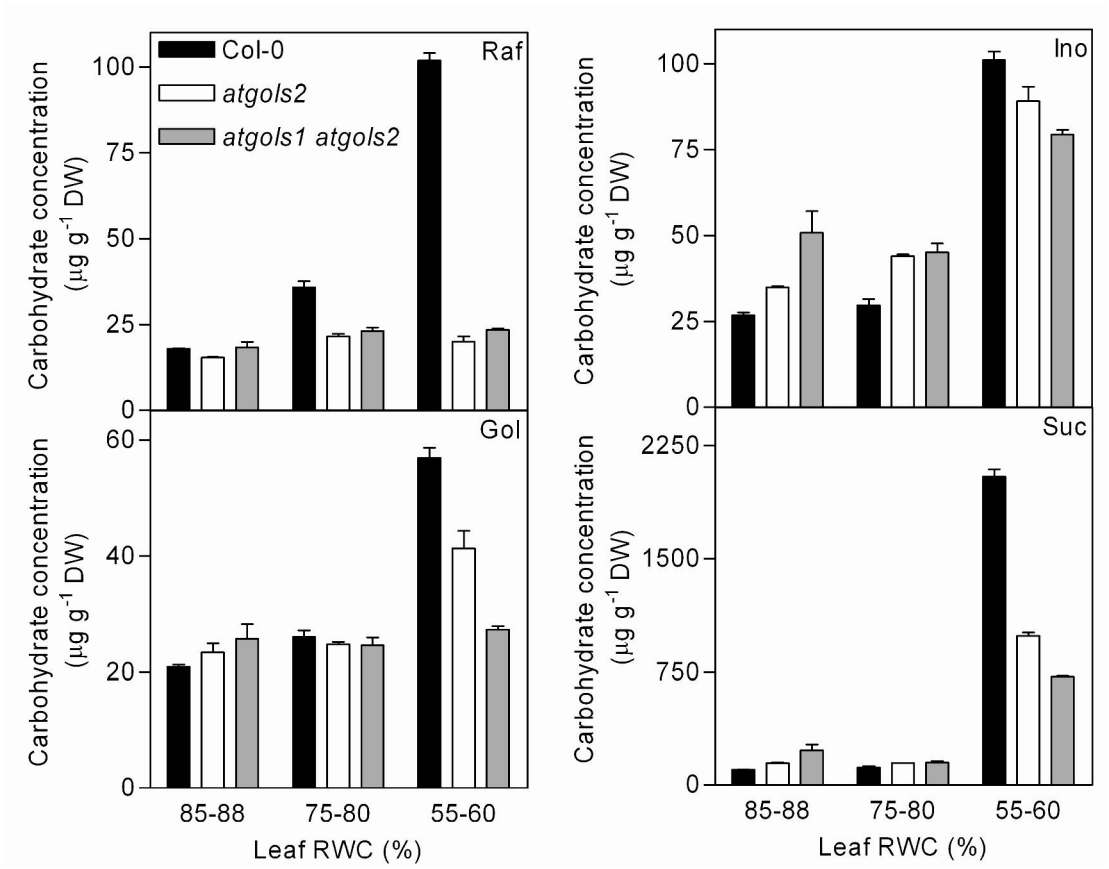


**Figure 3.5**: Changes in WSCs in the leaves of Arabidopsis wild type plants and a*tgols* T-DNA insertion mutants challenged with water deficit. Leaf relative water contents (RWC) of between 75-80% represent a 5 d period of water deficit. Stressed (S) plants represent a 7 d water deficit, when *atgols2* and *atgols1 atgols2* plants showed a visble loss of leaf turgor and represents leaf RWCs of 53 and 60%, respectively. Col-0 plants (S) represent a 9 d water deficit when these plants showed visible loss of leaf turgor and represents a leaf RWC of 63%. Data points represent the means±SE of six replicates. FT, Full turgor; Raf, raffinose; Gol, galactinol; Ino, *myo*-inositol; Suc, sucrose.

## 3.4 DISCUSSION

Because a functional role for *AtGolS1* was recently described in the heat shock response of Arabidopsis (Panikulangara et al., 2004; Nishizawa et al., 2006; Nishizawa-Yokoi et al., 2009) and *AtGolS2* is the most strongly transcriptionally upregulated isoform in Arabidopsis leaves under conditions of water deficit and high salinity (Taji et al., 2002), we opted for a strategy of using a previously described *atgols1* T-DNA insertion mutant (Nishizawa et al., 2008) to generate a double mutant carrying T-DNA insertions in both *AtGolS1* and *AtGolS2* (*atgols1 atgols2* plants; Fig. 3.1A). Together with the *atgols2* T-DNA insertion mutant, we used this double mutant to examine the functional contribution of these two GolS isoforms in water deficit-induced Gol and Raf accumulation in the leaves of these plants.

### 3.4.1 Water deficit induces GolS activity increases in leaves

Despite a clear transcriptional up-regulation of *AtGolS1* in the leaves of water deficit-stressed (55-60% RWC) *atgols2* plants, GolS activity increased only marginally (3-fold) when compared to activity in the leaves of water deficit-stressed (55% RWC) Col-0 plants, which increased 20-fold. Furthermore, GolS activity in water deficit-stressed leaves of *atgols1 atgols2* plants was marginally lower (2-fold, 0.17 nkat $g^{-1}$ DW) than in *atgols2* plants, suggesting that the difference of about 0.2 nkat $g^{-1}$ FW represents the contribution of the AtGolS1 isoform during water deficit. Our HPLC-PAD data demonstrated that Gol concentrations in water deficit-stressed leaves from *atgols1 atgols2* plants were 1.6-fold lower than that in *atgols2* plants. Collectively, our data suggests that AtGolS1 is an insignificant contributor to water deficit-induced Gol and Raf accumulation in Arabidopsis leaves and that it is, in fact, the AtGolS2 isoform that is responsible for the bulk of water deficit-induced Raf accumulation in leaves. Recent evidence in support of our conclusion is a demonstration that *AtGolS1* is a *bona fide* target of the HSF3 heat shock transcription factor and that *atgols1* plants fail to accumulate heat stress-induced Gol and Raf (Panikulangara et al., 2004), suggesting that *AtGolS1* may be the principle GolS isoform responsible for heat stress-induced Gol and Raf accumulation in Arabidopsis leaves. An interesting observation was the clear occurrence of *AtGolS1* transcripts in the leaves of *atgols2* plants at full turgor, and a higher transcriptional increase in water deficit-stressed leaves of *atgols2* plants compared to Col-0 (Fig 3.1 B). Despite this, *atgols2* plants are clearly hypersensitive to water deficit and do not show any water deficit-associated increases in either GolS activity (Fig 3.4) nor Gol and Raf accumulation (Fig 3.5).

### 3.4.2 Gol, Raf and Suc accumulation is attenuated in water deficit-stressed leaves of *atgols* mutant plants

GolS activity increased 20-fold in water deficit-stressed leaves (55-60% RWC) of Col-0 plants, correlating to an increase in Gol (2.7-fold) and Raf (5.7-fold) over an 8 d period of water deficit (Figs. 3.4 and 3.5) when Col-0 plants showed the first visible signs of leaf turgor loss. Concomitant to the absence of water deficit-induced GolS activity in stressed leaves of *atgols2* and *atgols1 atgols2* plants at 7 d water deficit (when these plants showed the first visible signs of leaf turgor loss; Fig. 3.2), Gol concentrations were attenuated to 1.5 and 2.6-fold, respectively, in water deficit-stressed leaves compared to those of Col-0 plants. Both *atgols2* and *atgols1 atgols2* plants also failed to accumulate Raf in water deficit-stressed leaves (55-60% RWC; Fig. 3.5). Arabidopsis is typically defined as a glycophyte in its response to water deficit and high salinity. In the context of our study, we were thus not looking for water deficit tolerance but an enhanced sensitivity in mutant plants. We consider that, although the sensitive response of wild type plants occurs 1 d later than that of the mutant plants, this remains a significant finding - particularly since the T-DNA insertions in the mutant plants result in severe attenuation water deficit-induced accumulation of both Gol and Raf, leading to a distinct water deficit stress-sensitive phenotype (Fig. 3.2) that we are able to correlate with our physiological data.

A surprising finding was that Suc accumulation was also severely attenuated in water deficit-stressed leaves of mutant plants compared to those of Col-0 plants (2.0 and 2.8-fold lower in *atgols2* and *atgols1 atgols2* plants, respectively; Fig. 3.5). We suggest two possible explanations for this observation. First, Suc is a substrate for Raf biosynthesis and thus, in the absence of a functional Gol biosynthetic pathway, the accumulation of Suc may be somehow reduced in *AtGolS* mutant plants to compensate for the lack of a complete Raf biosynthetic pathway. Alternatively, it has been proposed that acummulation of Suc and Raf are critical to the development of desiccation tolerance in seeds (Chen and Burris, 1990; Blackman et al., 1992). It has also been proposed that Suc-to-Raf mass ratios are of critical importance in the development of desiccation tolerance (Horbowicz and Obendorf, 1994). A recent report has demonstrated that Suc:Raf mixtures do indeed improve the desiccation tolerance of pea embryo protoplasts (Halperin and Koster, 2006). It is thus possible that despite Arabidopsis being a glycophyte, the mechanisms underpinning Suc and Raf accumulation for protection from desiccation are inherent. In our study, the Suc-to-Raf mass ratio in water deficit-stressed leaves of Col-0 plants was 20:1, but these plants still showed visible loss of leaf turgor after 8 d of water deficit. Interestingly, in

water deficit-stressed leaves of *atgols2* and *atgols1 atgols2* plants, this ratio was much higher (about 49:1 and 31:1, respectively) and these plants showed visible loss of leaf turgor after 7 d of water deficit (Fig. 3.3). It is thus possible that the Suc attenuation we observed in water deficit-stressed leaves of these mutant plants is representative of an adjustment toward a naturally optimal Suc-to-Raf mass ratio in the absence of Raf, and that collectively this contributes to the striking water deficit hypersensitive phenotype we observed.

However, in the absence of a second independent T-DNA line in this study, we are unable to confirm that the Suc attenuation we observed in *atgols2* plants is not indicative of a pleiotropic effect caused by an unmapped T-DNA insertion elsewhere in the genome. To address this, we have recently screened a newly available salk line (SALK_101144) for *AtGolS2* and have designated homozygous plants *atgols2-2*. More interestingly, we have preliminary evidence for the *atgols2* plants used in this study, showing that Pro accumulation in water deficit-stressed leaves is also severely attenuated compared to the water deficit-stressed leaves of Col-0 plants (data not shown). If these results will be confirmed in upcoming experiments and also will occur in *atgols2-2* plants, this would implicate at least Gol in a regulatory role, since we have shown that during water deficit, the *AtP5CS* gene behaves transcriptionally as in Col-0 plants (Fig. 3.1B). Such a conclusion may not be unwarranted as recent findings have placed Gol as a critical signalling component in the acquisition of induced systemic resistance during pathogen infection (Kim et al., 2008).

The constitutive overexpression strategy of *AtGolS2* described for Arabidopsis (Taji *et al.* 2002) is sufficient to demonstrate that hyper-accumulation of a compatible solute imparts water deficit tolerance. However, many other examples of compatible solute engineering have reported enhanced stress tolerance such as glycine betaine (Alia et al., 1998; Holmstrom et al., 2000; Huang et al., 2000), proline (Nanjo et al., 1999; Hong et al., 2000), mannitol (Tarczynski et al., 1992; Thomas et al., 1995) and trehalose (Holmstrom et al., 1996; Han et al., 2005). Using transgenic plants that hyper-accumulate a particular compatible solute to elucidate a functional role for that solute in abiotic stress tolerance is challenging, particularly given that by definition compatible solutes can occur at high intra-cellular concentrations without any deleterious effects to the plant. Thus, overexpression of *AtGolS2* in Arabidopsis may arguably result in enhanced osmotolerance because hyper-accumulation of Gol and Raf exerts a concentration-related osmotic effect. Using the reverse genetic approach reported here, analysing Arabidopsis *GolS* T-DNA insertion mutants, we

have been able to explicitly demonstrate that the absence of an active water deficit-induced AtGolS2 isoform led to severe attenuation of Gol and Raf accumulation in the leaves of water deficit-stressed mutant plants. Most importantly, in the absence of these solutes, mutant plants displayed a water deficit hypersensitive phenotype, losing leaf RWC and turgor more rapidly than water deficit-stressed Col-0 plants, suggesting that Gol and/or Raf may function as typical compatible solutes providing a certain degree of osmotolerance to Arabdopsis leaves exposed to water deficit.

In conclusion, we have demonstrated using a double T-DNA insertion mutant for *AtGolS1* and *-2*, that water deficit-induced Raf accumulation in the leaves of Arabidopsis is mainly due to the activity of the AtGolS2 isoform. Furthermore, the absence of this activity in an *AtGolS2* T-DNA insertion mutant (*atgols2*) results in severe attenuation of Gol and Raf accumulation in the leaves of water deficit-stressed plants and leads to a striking hypersensitive phenotype in these mutant plants. To our knowledge, this is the first report showing that the removal of water deficit-induced GolS genes from vegetative tissue results in an increased sensitivity to water deficit. However, Suc accumulation is also severely attenuated in the leaves of water deficit-stressed *atgols2* and *atgols1 atgols2* plants. Since Suc is also suggested to be a more or less universal stress protectant, it is unclear if the hypersensitive phenotype we observed is the results of perturbation of Raf accumulation alone or a combined effect of both Suc and Raf accumulation. Furthermore, preliminary findings suggest that Pro accumulation is also attenuated in the leaves of water deficit-stressed *atgols2* and *atgols1 atgols2* plants, necessitating more comprehensive analyses that would include the second independent T-DNA insertion line *atgols2-2*.

# Chapter IV: Manipulation of RFO concentrations *in vivo*, identification and functional expression of Arabidopsis *ATSIP2* as a Raf specific alkaline $\alpha$-Gal

## 4.1 INTRODUCTION

Over-expression strategies, using the first dedicated RFO biosynthetic enzyme GolS, have been employed to manipulate RFO concentrations *in vivo* (Taji et al., 2002; Zuther et al., 2004). In such approaches, the use of the cauliflower mosaic virus 35S (CaMV) promoter results in constitutive overexpression of a *GolS* cDNA and hyperaccumulation of Gol and Raf. Transgenic Arabidopsis constitutively overexpressing the *AtGolS2* gene were reported to hyperaccumulate Raf to concentrations of between 200-400 $\mu g\ g^{-1}$ FW, in the absence of abiotic stress (Taji et al., 2002). Similarly, overexpression of a *Cucumis sativus* GolS in Arabidopsis was reported to result in a Raf mass increase of about 20-fold (120 $\mu g\ g^{-1}$ FW) in transgenic plants (Zuther et al., 2004). A novel strategy, describing the silencing of an $\alpha$-Gal in petunia reported anti-sense lines hyperaccumulating Raf up to 1.7 $mg\ g^{-1}$ FW, 22-fold higher than in wild type plants (Pennycooke et al., 2003)

In keeping with the theme of this thesis, analysing the effects of RFOs in abiotic stress tolerance, we were interested in an experimental methodology that would allow Arabidopsis plants to naturally accumulate abiotic stress-induced Raf, but to remove this stress-induced Raf at the point of accumulation. To this end, we envisaged a strategy that would include the constitutive over-expression of an $\alpha$-Gal in Arabidopsis. We thus wished to (i) keep the RFO biosynthetic pathway intact, (ii) have $\alpha$-Gal overexpressing transgenic lines that would attenuate Raf accumulation to varying degrees upon stress treatment and (iii) analyse the stress performance of these transgenic lines, correlating performance to Raf concentration.

Arabidopsis acidic and alkaline $\alpha$-Gals are poorly described. Recent analysis of the Arabidopsis cell wall proteome has identified a number of proteins which are similar to acidic $\alpha$-Gals (Hye-Kyoung et al., 2005; Bayer et al., 2006) and they have been proposed to function in leaf development by degrading cell wall polysaccharides, thereby loosening the cell wall and allowing cell expansion (Chrost et al., 2007). The alkaline $\alpha$-Gals were identified when a group of functionally unidentified seed imbibition proteins (SIP) were observed to increase in germinating barley seed embryos (Heck et al., 1991). The cloning and functional expression of two homologous SIP cDNAs from melon fruit showed that they displayed distinct $\alpha$-Gal activity at alkaline pH, suggesting that SIP proteins are likely to represent alkaline $\alpha$-Gals in plants and revealing a previously unknown family of glycosyl hydrolases

(Carmi et al., 2003).They were subsequently identified to be present in a number of other plant species including Arabidopsis, which contains two annotated *SIP* genes (*ATSIP1*, At1G55740; *ATSIP2*, At3G57520).

The *ATSIP1* gene has been previously characterised using a reverse genetic approach analysing T-DNA insertion mutants. ATSIP1 was reported to share an 81% amino acid similarity with a *Cucumis* RafS (Anderson and Kohorn, 2001). In that study the absence of a functional ATSIP1 led to *atsip1* plants accumulating less Suc, and Ver. Furthermore, *atsip1* plants were insensitive to exogenous application of Raf whilst wild type plants were not, allowing the authors to indirectly conclude that *ATSIP1* encoded an enzyme involved in RFO biosynthesis in Arabidopsis.

Since ATSIP1 had been partially characterised and suggested to be a synthase involved in RFO biosynthesis, we undertook to functionally express and characterise the *AtSIP2* gene (At3g57520), which shows 76% amino acid similarity to the melon $\alpha$-Gal *CmAGA2* (genbank acc: AY114165) that was functionally demonstrated to be a Raf specific alkaline $\alpha$-Gal (Gao and Schaffer, 1999). Furthermore, if this proved to be a *bona fide* alkaline $\alpha$-Gal, it was to be used in the over-expression strategy described above. This work was conceptualised and initiated by myself but is currently part of an on going collaboration with Ph.D. candidate Aurélie Egert.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Heterologous expression of AtSIP2

#### 4.2.1.1 Riken cDNA clones

The Arabidopsis *AtSIP2* gene (At3g57520) was obtained as a full length cDNA from the Riken Arabidopsis full length clone database (pda02775, www.brc.riken.jp). The cDNA was amplified using the Expand High Fidelity PCR System (Roche, Basel, Switzerland) according to the manufacturers instructions, using open reading frame specific primers (*AtSIP2*$_{fwd}$ 5' ATGACGATTACATCAAATATCTCTG, *ATSIP*2$_{rev}$ 5' CTAGACCAGAATCTCAACATG) at a primer annealing temperature of 58°C. The resulting product was purified using the Wizard® SV Gel and PCR Clean-Up System (Promega AG, Dübendorf, Switzerland) according to the manufacturers instructions, cloned into the pGEM®-T Easy vector (Invitrogen Basel, Switzerland) and transformed into *E. coli* (DH10b) following the manufacturer's instructions. After sequence verification this was utilised as the source for sub-cloning into the pFastBac vector system (Invitrogen).

#### 4.2.1.2 Cloning into pFastBac 1 and bacmid generation

The *ATSIP2* cDNA was sub-cloned by standard restriction digest and ligation reactions, from pGEM®-T Easy into the pFastBac HTc vector (Invitrogen), using the *Not*I restriction endonuclease. Ligation reactions (*ATSIP2*::pFastBac HTc) were transformed into *E. coli* (DH10b) using a conventional heat shock method and plated onto Luria agar (LA) plates supplemented with the appropriate antibiotics ($^{100}$Amp). After 18 h incubation at 37°C, colonies were screened for 5'-3' orientation of *AtSIP2* using a pFastbac HTc forward primer (5' ATTCATACCGTCCCACCATC) and the *ATSIP*2$_{rev}$ primer, in a standard 50 µL PCR reaction at a primer annealing temperature of 58°C. Plasmid DNA was isolated from the appropriate colonies and transformed into *E. coli* (DH10bac, Invitrogen) for bacmid generation, following the manufacturers protocol (bac to bac manual, Invitrogen). Colonies transformed with *ATSIP2*::pFastBac HTc were selected for on LA plates supplemented with the appropriate antibiotics ($^{50}$Kan, $^{7}$Gent, $^{10}$Tet) and spread with 40 µL Blue-White Select™ screening reagent (Sigma, Switzerland). Plates were incubated at 37°C for 48 h. Positive colonies (white) were subsequently patched onto new LA plates (as described above) and incubated at 37°C for a further 24 h to confirm that they were

not false positives. Colonies were then innoculated into 5 mL LB ($^{50}$Kan, $^{7}$Gent, $^{10}$Tet) and grown overnight at 37°C (with agitation). Bacmid DNA was isolated following the manufacturer's instructions outlined in the bac-to-bac manual (Invitrogen), aliquoted (7 µL) and stored at -20°C for future use in baculovirus generation. Aliquots (5 µL) were also tested for gene transposition, using M13 (forward and reverse) and gene specific primer combinations.

### 4.2.2 *Spodoptera frugiperda* (Sf9)cells

Cells were cultured in a humidified growth chamber at 27°C, in 10 mL BD, BaculoGold™ TNM-FH insect cell medium (BD Biosciences, Ontario, Canada) supplemented with penicillin (90 U $mL^{-1}$) and streptomycin (90 µg $mL^{-1}$) for all manipulations described, unless otherwise stated. Cells were sub-cultured every 72 h by splitting the culture volume equally into two polystyrene cell culture dishes (10x2 cm, Corning Incorporated, NY, USA) and aliquoting the growth medium described above to a total volume of 10 mL per dish. Unsupplemented Graces Insect cell medium (Invitrogen, no fetal calf serum and no antibiotics) was used for exclusively for transfection procedures.

### 4.2.3 Transfection of SF9 Cells

#### 4.2.3.1 Generation of virus stock

Sf9 cells and media were pooled from three culture dishes into a 50 mL sterile falcon tube and subsequently centrifuged (500 *g*, 5 min). The supernatant was aspirated and the cell pellet re-suspended in 10 mL unsupplemented Graces insect cell medium. Cell density was determined, and the cells diluted to 9 x $10^5$ cells $mL^{-1}$. Aliquots (1 mL) were dispensed into the wells of a microtiter plate (2x3 well format, 0.35 cm ∅) and incubated at room temperature for 1 h to facilitate cell attachment. For each well, one aliquot (7 uL) of bacmid was thawed on ice and 100 µL of unsupplemented Graces insect cell medium added. In a separate Eppendorf tube 6 µL of Cellfectin reagent (Invitrogen) and 100 µL of unsupplemented Graces insect cell medium were combined, transferred into the Eppendorf tube containing the bacmid preparation and incubated at room temperature for 45 min. Medium was then aspirated from each well after which 0.8 mL of unsupplemented Graces insect cell medium was added to each of the bacmid preparations. Medium was carefully aspirated from each of the wells and the bacmid preparation overlayed onto the cells,

after which the plate was incubated at 27°C for 5 h. The medium was then aspirated and replaced with 2 mL of BaculoGold™ TNM-FH medium supplemented with antibiotics. After 72 h incubation, the medium from three wells was collected into sterile 15 mL tubes, clarified by centrifugation (500xg, 5 min) and stored at 4°C protected from light. This represented the $P_0$ viral stock.

Assuming a viral titer of $2x10^7$ pfu $mL^{-1}$ for $P_0$, viral stocks were amplified by infecting, at a multiplicity of infection (MOI) of 0.2, a culture dish seeded with $5x10^6$ cells in 10 mL BaculoGold™ TNM-FH media. Infected cells were incubated for 48 h and the medium then collected, clarified by centrifugation as described above and used in further amplification. After each round of amplification it was assumed that the viral stock was amplified 10-fold. For assaying recombinant protein expression, $P_2$ viral stocks ($\cong 2 \times 10^8$ pfu $mL^{-1}$) were used to infect *SF9* cells as described below.

### 4.2.4 Recombinant protein expression and enzyme assays

Sf9 cells were seeded at $5x10^6$ cells, into culture dishes containing 10 mL BaculoGold™ TNM-FH medium supplemented with antibiotics, and incubated at room temperature for 1 h. Baculovirus stock (P2) was added to the medium at MOI 7.5 and the plates incubated for 48 h at 27°C. Cells were then collected from three infected plates, pooled into a 50 mL sterile Falcon tube and centrifuged (500 g, 5 min). After aspirating the supernatant, the cell pellet was re-suspended in 2mL extraction buffer [100 mM Hepes-KOH, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM DTT, 1 mM Benzamidine, 1 mM PMSF, 0.05% (v/v) Triton X-100]. Cells were lysed using a potter connected to an electric drill and centrifuged (12000 *g*, 4°C, 10 min). This clarified crude extract was transferred into a sterile 2 mL Eppendorf tube. Control samples represented uninfected SF9 cells that had been treated in the same manner described above.

Aliquots (150 μL) of clarified crude extract were centrifuge desalted by gel filtration (1400 *g*, 4°C, 2 min) through 5 mL Sephadex G-25 columns (fine, final bed volume of 3 mL). Columns were pre-equilibrated with assay buffer (100 mM Hepes-KOH, pH 7.5). Pre-equilibration was performed twice with 2 mL of assay buffer. Enzyme activity assays were conducted in a total volume of 70 μL containing 35 μL clarified crude extract and 35 μL of assay buffer (100 mM Hepes-KOH, pH 7.5, 100 mM Raf), at 30°C for 1 h. The final Raf concentration in all assays was 50 mM. Clarified crude

extracts were also assayed for RafS activity as described except that assay buffer contained 100 mM Suc and 10 mM Gol. Samples were desalted for HPLC-PAD analysis as previously described and analysed on the BC-100 chromatographic system (Peters et al., 2007; Peters and Keller, 2009).

### 4.2.5 Deoxygalactonojirimycin (DGJ) inhibition

Clarified crude extract was incubated at room temperature for 10 min, in the presence of 10 μM DGJ. Samples were then assayed for α-Gal activity as described above.

### 4.2.6 pH optimum

For the determination of the pH optimum of recombinant ATSIP2, crude extracts were prepared from Sf9 cells as described above. The following buffers were used in the determination, full strength McIlvaine buffer (pH 5.0, 5.5 and 6.0), 100 mM MES-KOH buffer (pH 6.0, 6.5, 7.0), 100 mM Hepes-KOH buffer (pH 7.0, 7.5, 8.0). All buffers contained 62.5 mM Raf. Enzyme activity assays were conducted in a total volume of 50 μL containing 10 μL of clarified crude extract and 40 μL of assay buffer. The final concentration of Raf in each assay was 50 mM.

## 4.3 Overexpression of ATSIP2 in Arabidopsis

### 4.3.1 Gateway cloning strategy:

The destination vectors pMDC32 (Curtis and Grossniklaus, 2003) and pCB2010 (Lei et al., 2007) were chosen to generate plant over-expression constructs. A high fidelity PCR product of *AtSIP2* was cloned in the pCR®8 vector using the pCR®8/GW/TOPO®TA Cloning Kit (Invitrogen) following the manufacturer's instructions. Antibiotic resistant colonies ($^{100}$Spec.) were subjected to a colony PCR using the *ATSIP*2$_{rev}$ primer and the GW1 pCR®8 specific primer (5' GTTGCAACAAATTGATGAGCAATGC) to identify colonies where *AtSIP2* had inserted into pCR®8 in 5'-3' orientation. Plasmid minipreparations were obtained from postive colonies and used in an LR clonase™ reaction (Invitrogen) to obtain *AtSIP2*::pMDC32 and *AtSIP2*::pCB2010. Clonase reactions were transformed in E.coli (DH10b) using a conventional heat shock method. Antibiotic resistant colonies ($^{50}$Kan.) were subjected to a PCR using a pMDC32 specific primer (5' AGAGGATCCCCGGGTACC) and the *AtSIP*2$_{rev}$ primer, to confirm that *AtSIP2* had transposed into the vector. Plasmid minipreparations were obtained from positive colonies, and used in *Agrobacterium* transformations.

### 4.3.2 Plant transformation:

#### 4.3.2.1 *Agrobacterium tumefaciens* transformation:

Plasmid minipreparations of *AtSIP2*::pMDC32 were transformed by electroporation into *A. tumefaciens* (GV3101), using a Genepulser® (2.5 kV; 100 Ω; 25 µF, Bio-Rad). Transformed colonies were selected for on LA plates supplemented with the appropriate antibiotics ($^{50}$Rif., $^{25}$Gent., $^{50}$Kan.) after incubation at 27°C for 48 h.

#### 4.3.2.2 Plant Material and growth conditions:

Arabidopsis (Col-0) seeds were stratified for 48 h at 4°C and planted (5 seeds to a pot) in commercial potting soil (Einheitserde, type ED73, Gebr. Patzer GmbH & Co. KG, Schopfheim, Switzerland). Pots were maintained in a controlled environment chamber (8 h light, 120 µmol photons $m^{-2}$ $s^{-1}$, 22°C, 8 h dark, 60% RH) for approximately 8 weeks, until the plants had developed multiple inflorescences. They were then used for floral dip transformations as described below.

#### 4.3.2.3 Plant transformation and selection:

Plants were transformed with *A. tumafaciens* (GV3101) harbouring the *AtSIP2*::pMDC32 construct, using the floral dip transformation method (Clough and Bent, 1998), with minor modifications. Single *Agrobacterium* colonies were innoculated into 5 mL LB cultures supplemented with the appropriate antibiotics ($^{50}$Rif., $^{25}$Gent., $^{50}$Kan.), 5mM Acetosyringone and grown for 16 h at 27°C, with agitation. This culture was used as an inoculum for 100 ml pre-warmed (27°C) LB containing acetosyringone and antibiotics as outlined, and grown as described for 16 h. Subsequently, the culture was centrifuged (7000 *g*, 10 min) and the pellet resuspended in 5% (w/v) Suc. Plants were dipped for 5 min and the procedure was repeated 4 d later.

Seeds (T1) were collected, surface sterilised and plated onto MS plates supplemented with 5% (w/v) Suc and $^{25}$Hyg. Selection for hygromycin resistant individual plants was conducted as previously described (Harrison et al., 2006). All resistant plants were transferred to soil and maintained in the controlled environment chamber described above. Seeds (T2) were collected and plants representing the T2 generation used for further characterisation.

#### 4.3.2.4 Characterisation of transgenic lines:

Plants representing the T2 generation after transformation were tested for overexpression of *ATSIP2* using semi quantitative PCR as outlined in chapter 3 (3.XX). After testing the linearity of an actin gene as a constitutively expressed control, RT-PCRs were conducted on first strand cDNA using an annealing temperature of 56°C and 24 cycles. Lines were chosen with high, moderate and low over-expression, relative to an untransformed control plant (designated Ox-AtSIP2 1.1 to 1.3, respectively).

Alkaline α-Gal activity was measured in these lines by homogenising 200 mg leaf tissue with 400 µL extraction buffer [50 mM Hepes/KOH pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 20 mM DTT, 0.1% (v/v) Triton X-100, 1 mM benzamidine, 1 mM PMSF, 50 mM Na-ascorbate, 2% (w/v) PVP]. After centrifugation (12000 *g*, 10 min), 100 µL of homogenate was desalted by gel filtration and crude extracts used to assay for α-Gal activity as described for recombinant expression assays (above).

## 4.4 RESULTS

### 4.4.1 Functionally expressed plant alkaline α-Gals share identities with AtSIP2

A BLASTp search (www.ncbi.nlm.nih.gov/BLAST/) indicated that AtSIP2 shared identity to plant sequences encoding both putative and functionally confirmed alkaline α-Gals. An alignment of AtSIP2 against these functionally confirmed α-Gals revealed that the protein shared the highest amino acid similarity (77%) with *Pisum sativum* (PS II) and the lowest (60%) with *Zea mays* (Zm II). A 76% amino acid similarity was evident with a Raf specific α-Gal isoform from *Cucumis melo* (Cm II, Fig. 4.1).

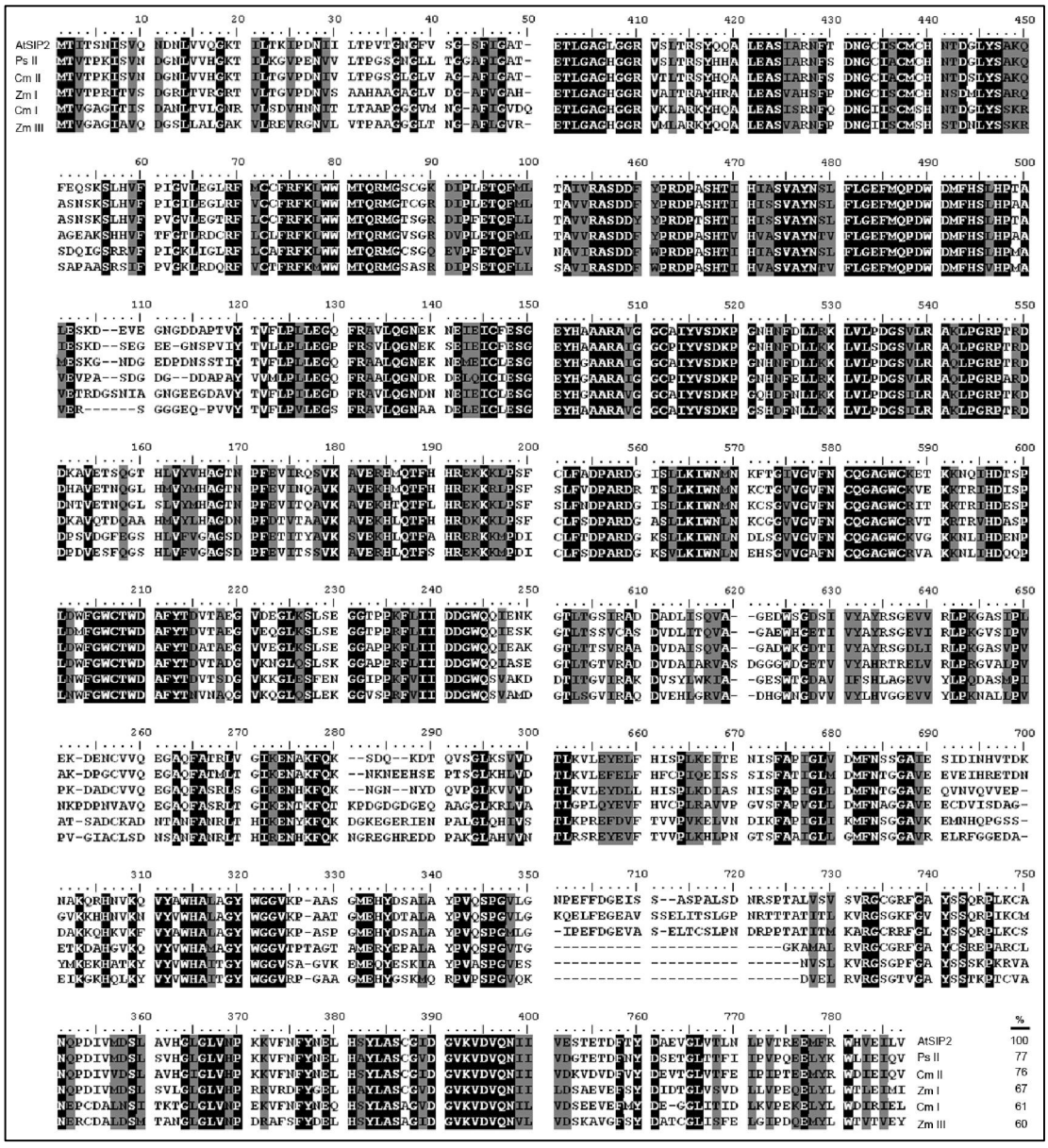


**Figure 4.1**: Amino acid alignment of AtSIP2 against functionally expressed plant alkaline α-Gals from *Pisum sativum* (AJ630105), *Cucumis melo* (CmI AY114165, CmII AY114165)and *Zea mays* (ZmI AF497510,ZmIII AF497512. Identical amino acids are highlighted in black and similar ones in grey. Identities (%) are presented against the predicted amino acid sequence of AtSIP2.

### 4.4.2 Recombinant AtSIP2 is an alkaline α-Gal with a substrate preference for Raf

Crude cell extracts obtained from Sf9 cells infected with bacmids containing the *ATSIP2* cDNA, incubated in the presence of 50 mM Raf at pH 7.5, were able to hydrolyse Raf to Suc and Gal. This activity was neither detectable in crude extracts from uninfected Sf9 cells nor in the presence of the α-Gal inhibitor, DGJ, at a concentration of 10 μM in infected crude extracts (Fig. 4.1). No activity was observed when ATSIP2 was incubated in the presence of Suc and Gol, substrates for RafS. The pH optimum of recombinant ATSIP2 was determined to be between 7.5 – 8.0 (Fig. 4.2).

Further characterisation of the alkaline α-Gal activity of AtSIP2 indicated that the recombinant protein was able to cleave the artificial substrate *p*-nitrophenyl-alpha-D-galactopyranoside (pNPGal). Conversely, hydrolytic activity was not observed using the β-linked variant of pNPGal (Fig. 4.3 A). Recombinant ATSIP2 activity was inhibited in the presence of increasing Gal concentrations (Fig. 4.3 B). The recombinant protein displayed a distinct substrate preference for Raf with only minor activity observed on Sta and no activity detected on Gol (Fig. 4.4).

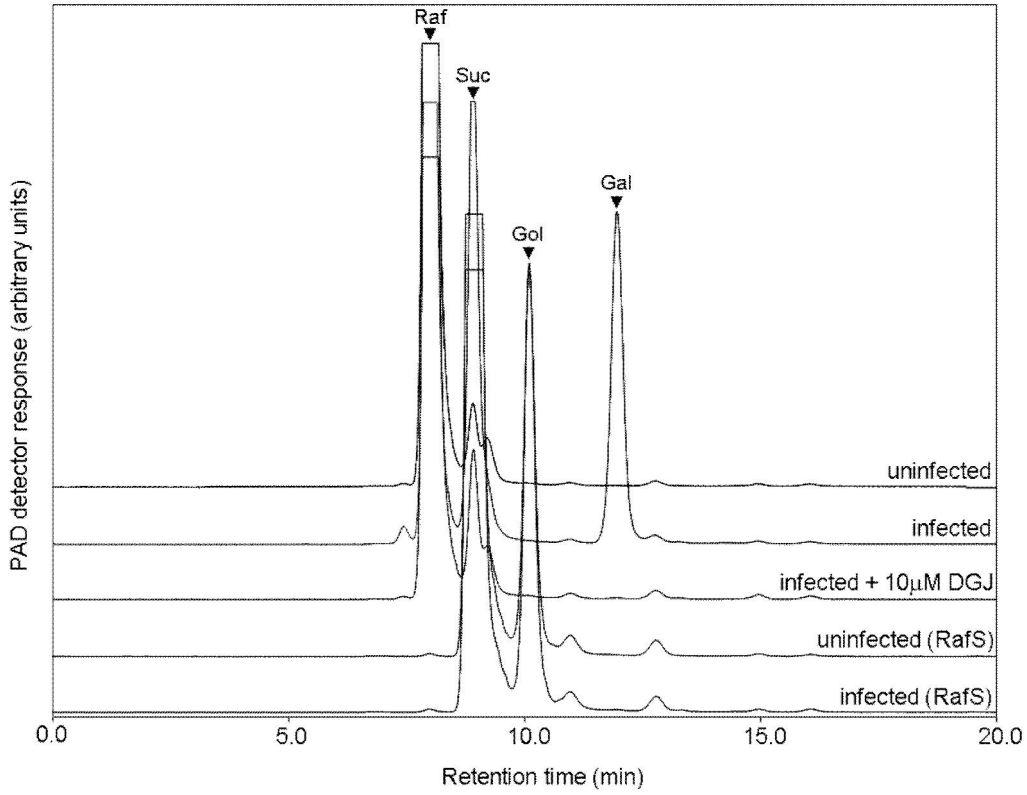


**Figure 4.1**: HPLC-PAD chromatogram of crude protein extracts obtained from uninfected and infected (AtSIP2 bacmid) Sf9 cells, incubated in the presence of (i) 50 mM Raf, (ii) 50 mM Raf + 10 μM DGJ and (iii) 50 mM Suc and 10 mM Gol (RafS), at a pH of 7.5.

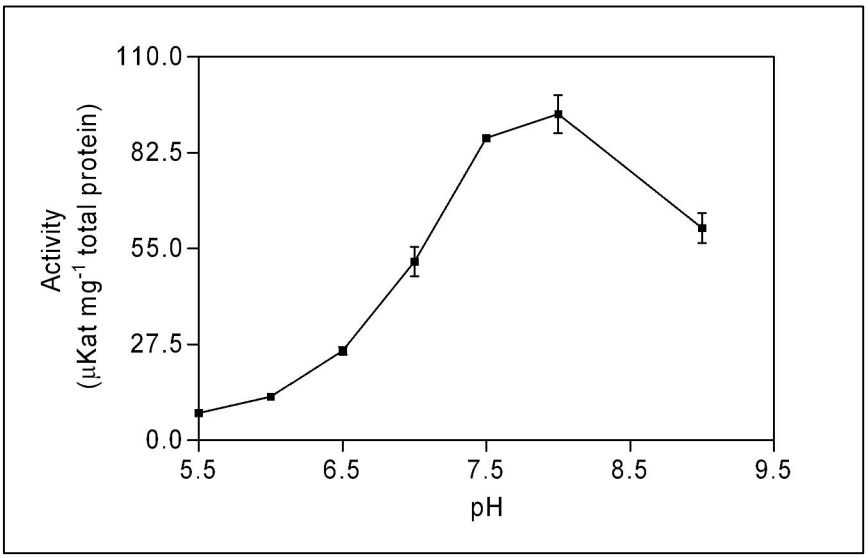


**Figure 4.2**: pH optimum of α-Gal activity measured in crude cell lysates from SF9 cells infected with the ATSIP2. Raf was used at a final concentration of 50 mM in the assays. Error bars represent the mean ±SE of six replicates. The overlapping values between the different buffers used over the pH range were omitted as they did not vary much.

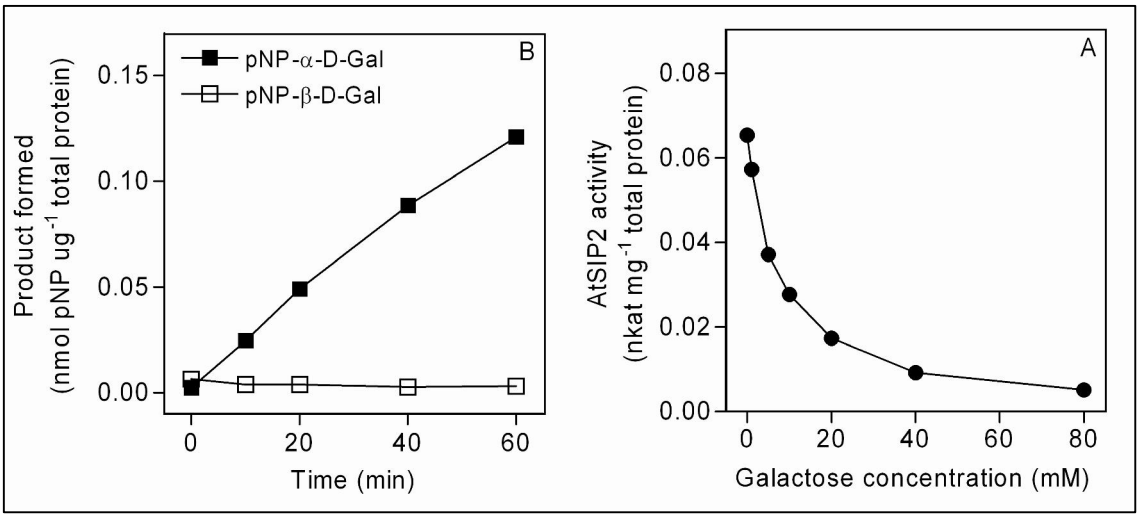


**Figure 4.3**: (A) Activity of recombinant ATSIP2 on the artificial substrate pNPGal (α and β) and (B) Gal inhibition of recombinant ATSIP2 activity. Each data point represents the mean ±SE of six replicates. Where no error bars are presented they were smaller than the data point. Figure courtesy of A. Egert.

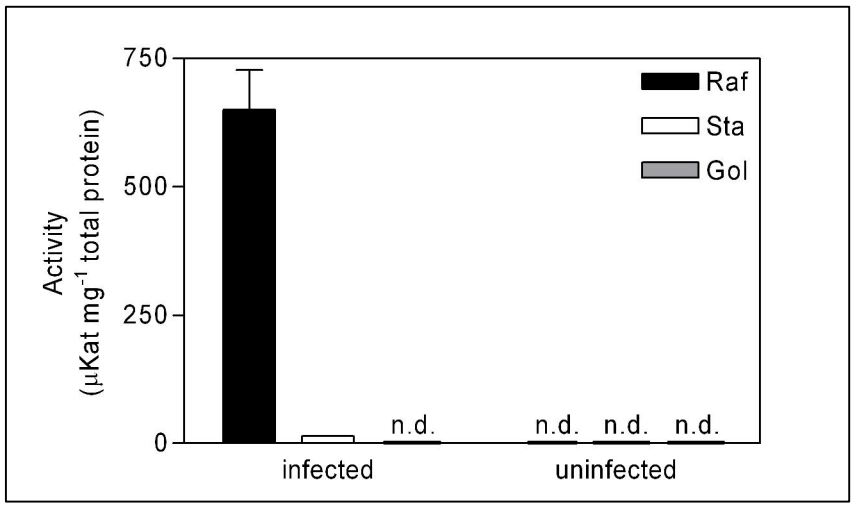


**Figure 4.4**: α-Gal activity of crude protein extracts obtained from uninfected and infected (*AtSIP2* bacmid) Sf9 cells and incubated in the presence of either 50 mM Raf, Sta or Gol, at a pH of 7.5. An absence of activity is denoted by n.d. (not detected). Errors bars represent the mean ±SE of six replicates.

### 4.4.3 Leaves of Ox-AtSIP2 plants show higher alkaline α-Gal activities

Hygromycin-resistant F1 plants were transferred to soil and tested for both *ATSIP2* expression (semi quantitative PCR) and alkaline α-Gal activity. ATSIP2 transcripts occurred in varying abundance in 8 independent transgenic lines. From these results, the 3 lines 1.1, 1.2 and 1.4 were chosen for analysis of alkaline α-Gal activity using Raf as a substrate. At pH 7.5, α-Gal activity of leaf crude extracts from the Ox-*ATSIP2* lines, were clearly higher than in Col-0 plants. Activity was 1.6-fold higher (1.3 nkat $g^{-1}$ FW) in *Ox-ATSIP2* 1.1, 1.1-fold higher in *Ox-ATSIP2* 1.2 (1.5 nkat $g^{-1}$ FW) and 0.7-fold higher in *Ox-ATSIP2* 1.4 (1.0 nkat $g^{-1}$ FW).

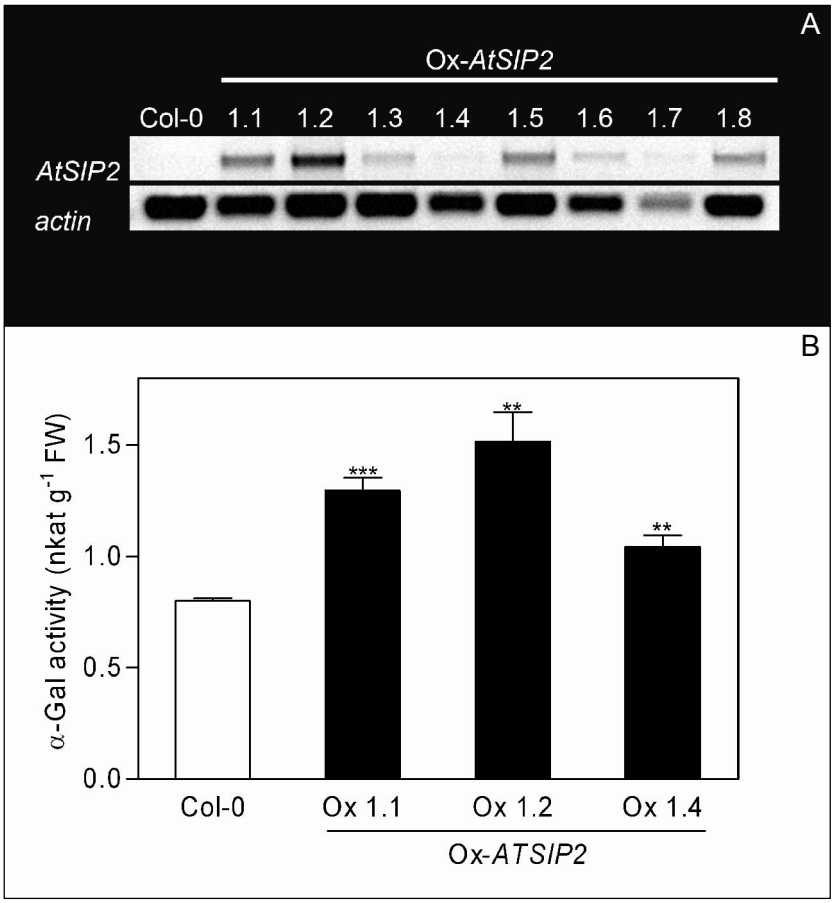


**Figure 4.5**: (A) Expression of *ATSIP2* in the leaves of 8 independent F1 Arabidopsis plants transformed with pMDC32::*ATSIP2* and (B) alkaline α-Gal activity in leaf crude extracts from corresponding Ox-*ATSIP2* lines 1.1, 1.2 and 1.4, incubated with 50 mM Raf at pH 7.5. Error bars represent the mean ±SE of six replicates. Statistical significance of a two tailed t-test is represented by stars (Ox 1.1, $p<0.0001$; Ox 1.2, $p<0.0015$; Ox 1.4, $p<0.0040$).

## 4.5 DISCUSSION

Plant α-Gals may not only be broadly characterised based on the pH optima of their activities (Keller and Pharr, 1996), but also on their substrate specificities. As mentioned, plant α-Gals have been implicated in a number of important physiological processes including RFO mobilisation during seed germination, cell growth through cell wall loosening, and phloem unloading in plants using RFOs as phloem translocates. We were interested in exploiting RFO (Raf) concentrations in Arabidopsis by manipulating the expression of an alkaline α-Gal.

Our initial approach of searching GenBank (www.ncbi.nlm.nih.gov/Genbank/) for Arabidopsis orthologues to functionally identified alkaline α-Gals identified the candidate genes, *ATSIP1* and *ATSIP2*, as putative alkaline α-Gals, based on sequence homology to functionally expressed alkaline α-Gals including two from *C. melo* fruit (*CmAGA1* and *-2*, Gao and Schaffer, 1999). Interestingly, *ATSIP1* has been studied via a reverse genetic approach using a T DNA insertion mutant (Anderson and Kohorn, 2001). The authors concluded it to be involved in RFO biosynthesis rather than catabolism. In that study, water-soluble carbohydrates were analysed by HPLC-PAD using a CarboPac PA1 column eluted isocratically with NaOH and the authors identified that RFOs were present in Arabidopsis leaves up to Ver. Our own routine HPLC-PAD analyses on WSCs from Arabidopsis has never identified RFO oligomers in leaf tissue beyond Raf. Similarly, other studies have not reported any RFO oligomer higher than Raf in Arabidopsis leaves, even under abiotic stress conditions (Taji et al., 2002; Zuther et al., 2004) nor have extensive metabolomic analyses on Arabidopsis identified RFOs beyond Raf (Kaplan et al., 2004; Wienkoop et al., 2008; Maruyama et al., 2009)

We focused effort on *ATSIP2* which was functionally uncharacterised and showed high similarity to *CmAGA2*, a Raf specific alkaline α-Gal (Gao and Schaffer, 1999) but which has been recently reported to be a RafS (Nishizawa et al., 2008; Maruyama et al., 2009). We chose the Sf9 insect cell system for heterologous expression as we determined in preexperiments that crude cell extracts of Sf9 cells showed no hydrolytic activity on Raf as a substrate. Conversely crude cell extracts from *E. coli* cultures did show a low and variable background hydrolytic activity on Raf making it an unsuitable system for biochemical characterisation of a recombinant α-Gal. We demonstrated conclusively that recombinant ATSIP2 was able to

hydrolyse Raf, but not Sta, *in vitro* (Fig. 4.1 and 4.4). Furthermore, using pNPGal (an artificial broad spectrum substrate for α-Gals) Aurélie Egert showed that recombinant ATSIP2 was only able to hydrolyse the α-linked and not the β-linked version of this substrate. We could also show that the ATSIP2 had a pH optimum of about 8 and was inhibited by increasing concentrations of Gal (Ki = 7.5 mM). Additionally, Raf hydrolase activity was completely abolished in crude cell extracts containing 10 μM of the α-Gal inhibitor DGJ (Fig. 4.1). Importantly, recombinant ATSIP2 was unable to synthesise Raf when incubated in the presence of Suc and Gol. Collectively, these data irrefutably demonstrate that AtSIP2 is a *bona fide* alkaline α-Gal with a substrate preference for Raf, very similar to its close orthologue *CmAGA2* which was also demonstrated to be Raf specific alkaline α-Gal (Gao and Schaffer, 1999).

The annotation of *SIP* genes defines them as having functional raffinose synthase domains (www.arabidopsis.org). We believe that many raffinose synthase genes may be erroneously annotated as alkaline α-Gals and *vice versa*. Support for this statement comes from two recent reports where real time PCR analysis was used to monitor changes in the expression profile of *ATSIP2* in Arabidopsis (Nishizawa et al., 2008; Maruyama et al., 2009). In both cases *ATSIP2* was defined as a *RafS*. However, the data we have presented clearly demonstrates that ATSIP2 is irrefutably an alkaline α-Gal and not a RafS.

Once ATSIP2 had been functionally identified, we generated two over-expression constructs where *ATSIP2* expression was constitutive (CaMV35S promoter, pMDC32, Curtis and Grossniklaus, 2003), or specific to the drought-inducible promoter of the *Rd29A* gene (pCB2010, Lei et al., 2007). We envisaged that we would then have two transgenic Arabidopsis systems where Raf accumulation was attenuated. Due to time constraints we were unable to bring these experiments to their final conclusion but have thus far demonstrated that F1 transgenic lines carrying pMDC32::*ATSIP2*, show higher gene expression levels and α-Gal activity (using Raf as a substrate) than wild type plants (Fig. 4.5). However, we used buffers at pH 7.5 for crude extracts and *in vitro* activity assays, and observed a relatively high alkaline α-Gal activity in Col-0 plants. This may be due to residual activity of acidic α-Gals in Arabidopsis crude extracts and these analyses are presently being repeated using other pH values (8.0 and 8.5). Despite this technical problem, selected transgenic lines still show higher α-Gal activities that are statistically significant.

Further characterisation of these transgenic lines where their stress performance is evaluated under water deficit and cold acclimation/frost tolerance are currently being undertaken by Ph.D. candidate Aurélie Egert. Other ongoing experiments borne from this initial work been presently conducted by Egert include the analysis of *ATSIP2* promoter GUS fusion constructs and the analysis of *ATSIP2* T-DNA insertion mutants (*atsip2-1*, *atsip2-2*) to identify a physiological role for this Raf-specific alkaline $\alpha$-Gal. To date, preliminary findings point to ATSIP2 functioning in two very different physiological aspects of Arabidopsis.

Firstly, using a 500 bp fragment of genomic DNA upstream of the *ATSIP2* start codon, I constructed a $\beta$-glucoronidase reporter gene fusion construct (pMDC 163, Curtis and Grossniklaus, 2003) and transformed this into Arabidopsis. GUS stains conducted, by Egert, on transgenic plants show very strong staining in the roots and sink leaves of seedlings, suggestive of a putative role in phloem unloading. Furthermore, Egert has definitively shown that the GUS-stain results correlate to *in vivo* $\alpha$-Gal activity in Arabidopsis. Using Raf as a substrate with crude enzyme extracts, she could demonstrate that activities were significantly higher in sink leaves and roots, compared to those observed in source leaves. Since Arabidopsis has been reported to mainly transport Suc in the phloem (Haritatos et al., 2000), we are pursuing this avenue using classical $^{14}CO_2$ photosynthetic pulse chase phloem exudation experiments to determine if Raf may also be transported in small amounts in the phloem.

Secondly, preliminary experiments have demonstrated that under cold-deacclimation from 4°C, the leaves of *atsip2* plants show no difference in the rate at which cold stress-induced Raf is degraded. However, 24 h after the relief of water deficit stress the leaves of *atsip2* plants show significantly higher Raf concentrations than wild type plants, suggesting that ATSIP2 may also function in degradation of water deficit-induced Raf upon relief of the stress. Both these observations hold the promise of very novel functions for this alkaline $\alpha$-Gal in Arabidopsis and Egert is presently completing repetitions of these experiments to confirm the findings.

# Chapter V: General summary, conclusions and outlook

## 5.1 General summary conclusions and outlook

Research into RFOs and their role/s in abiotic stress tolerance has undergone a recent resurgence. This is particularly due to the advent of large scale comparative transcriptomic and metabalomic analyses in Arabidopsis. A common feature of these analyses is that under multiple abiotic stresses in Arabidopsis, amongst the most statistically significant changes at (i) the transcriptomic level is an increase in GolS transcripts (Fowler and Thomashow, 2002; Kreps et al., 2002) and of (ii) the metabolomic level is an increase in Raf (Cook et al., 2004; Rohde et al., 2004; Kaplan et al., 2007). Such data indirectly suggest a crucial function for Raf in the abiotic stress tolerance response of Arabidopsis.

However, attempts to dissect these functional role/s *in vivo* have resulted in controversy, particularly in the cold acclimation/frost tolerance response of Arabidopsis, where Raf was suggested to have no functional contribution (Zuther et al., 2004). Furthermore, a pioneering study in Arabidopsis where *AtGolS2* was overexpressed resulted in transgenic plants hyperaccumulating Raf under normal growing conditions compared to untransformed plants. Transgenic plants displayed hypertolerance to water deficit. As we have described in this thesis (Chapter 3) these results only indirectly suggest that Raf is important to water deficit tolerance in Arabidopsis, particularly given that changes in gene expression, GolS activity and WSCs were not measured in transgenic plants subjected to water deficit.

In the work we have presented, we embarked on a multi-pronged approach to address functional contributions of RFOs in abiotic stress tolerance using the frost hardy Lamicacea *A. reptans*, which uses Sta as a phloem translocate and specialises in using multiple RFO oligomers as a carbon store. Furthermore, we examined the role of Raf in the water deficit tolerance response of Arabidopsis using a reverse genetic approach analysing T-DNA insertion mutants for GolS isoforms – assuming that if Raf was critical to stress tolerance then its absence in mutant plants would result in them being hypersensitive to the given stress. Finally, we employed a transgenic overexpression strategy to manipulate stress-induced Raf concentrations *in vivo*, using Arabidopsis. To this end we identified *ATSIP2* as a putative alkaline $\alpha$-Gal and through functional expression in Sf9 insect cells confirmed its identity as a Raf-specific alkaline $\alpha$-Gal. This gene was then over-expressed in Arabidopsis as a means of keeping the stress-inducible RFO biosynthetic pathway intact, but removing Raf as it accumulated, *in vivo*.

The major findings of this work are highlighted below,

### 5.2 Frost tolerance in excised leaves of the common bugle (*Ajuga reptans* L.) correlates positively with the concentrations of RFOs

We a previously described excised leaf system (Inan-Haab and Keller, 2002), that effectively uncouples RFO accumulation from low temperature acclimation demonstrating that excised leaves operate within normal photosynthetic parameters, producing $^{14}$C-Suc as the major photosynthate and transporting $^{14}$C-Sta, in the phloem as we have previously reported using *A. reptans* whole plants (Bachmann *et al.* 1994). As reported previously, RFOs increased in warm-incubated excised leaves (Inan-Haab and Keller, 2002). This total RFO increase correlated positively with a shift in the $EL_{50}$ value from about -10.5°C (control) to -24.5°C (30 DAE) showing for the first time quantitatively that, in *A. reptans* leaves, RFOs may improve frost tolerance irrespective of cold acclimation. The best correlation to the shift in $EL_{50}$ values was evident between concentration increases in higher RFO oligomers, which accounted for 65% of the total RFO increase.

We are presently pursuing a strategy to understand the *in vivo* role of higher RFO oligomers in frost tolerance. To this end we have created an over-expression construct (pMDC32::*ArGGT*), where the expression of the GGT cDNA is driven by a double CaMV 35S promoter. We envisage that Arabidopsis plants transformed with this construct may accumulate RFOs beyond Raf, under normal growing conditions. Transgenic plants will be subjected to frost tolerance experiments as described in this thesis to determine if they are more frost resistant than untransformed control plants. Given that GGT is localized to the vacuole, higher RFO oligomers accumulating in transgenic plants would most likely also occupy this compartment. Classical compartmentation studies will be conducted to confirm this, and such data should provide critical insight into how higher RFO oligomers may function as typical compatible solutes *in vivo*.

### 5.3 An Arabidopsis T-DNA insertion mutant for galactinol synthase 2 (*AtGolS2,* At1g56600) is hypersensitive to water deficit, failing to accumulate Gol, Raf and Suc

To further define the roles of GolS and its direct and indirect products with putative compatible solute characteristics, Gol and Raf, in water deficit tolerance, we used a T-DNA insertion mutant approach, downregulating the Raf biosynthetic pathway and

included the measurements of *AtGolS* expressions, GolS activities and water-soluble carbohydrate concentrations during stress. We opted for a strategy of using a previously described *atgols1* T-DNA insertion mutant (Nishizawa et al., 2008) to generate a double mutant carrying T-DNA insertions in both *AtGolS1* and *AtGolS2* (*atgols1 atgols2*). Together with the *atgols2* T-DNA insertion mutant, we used this double mutant to examine the functional contribution of these two GolS isoforms in water-deficit induced Gol and Raf accumulation in the leaves of these plants.

We could demonstrate that soil droughted *atgols2* and *atgols1 atgols2* plants lost water more rapidly than did wild type (Col-0) plants. This correlated to a pronounced loss of leaf turgor in the mutant plants that occurred a full day earlier than Col-0 plants. Water deficit-induced GolS activity and Gol and Raf accumulation was severely attenuated in the leaves of stressed mutant plants. A surprising finding was that Suc accumulation was also severely attenuated in the leaves of stressed mutant plants. As such the data set we have presented clearly demonstrates that water deficit-induced GolS activity is a prerequisite for Gol and Raf accumulation. However, it is unclear if the pronounced water deficit-sensitive phenotype we observed in the mutant plants is due to the attenuation of Gol and Raf or if this is representative of reduced Suc concentrations as well.

We have preliminary evidence that Pro concentrations are also severely attenuated in water deficit-stressed leaves of *atgols2* and *atgols1 atgols2* plants. These findings are astonishing as we could demonstrate that the water deficit induced transcriptional activation of the *AtP5CS* gene occurred comparably in both mutant and Col-0 plants. Clearly the regulation of compatible solute biosynthesis in Arabidopsis undergoing water deficit is complex, particularly given that Raf, Suc an Pro have long been suggested to be stress protectants and that their accumulation is severely attenuated in GolS mutant plants. We are presently looking toward establishing an academic collaboration, with the express purpose of comparatively analysing the changes in both the transcriptome and metabolome of *atgols2* and *atgols1 atgols2* plants undergoing water deficit. We believe that this would shed light on how the absence of functional water deficit-induced GolS transcripts and/or Gol and Raf concentrations affect other water deficit-induced compatible solute metabolic pathways in Arabidopsis. Futhermore, such comparitive analyses would by default include other stress related metabolic pathways that were not considered in this study.

## 5.4 Arabidopsis *ATSIP2* is an alkaline α-galactosidase with a substrate preference for raffinose

In order to manipulate stress-induced Raf concentrations *in vivo*, we compared the Arabidopsis sequence database to sequences of functionally expressed alkaline α-Gals. Two candidate genes, *ATSIP-1* and *-2* showed high homologies to the known alkaline α-Gals. Since *ATSIP1* had been partially characterised (Anderson and Kohorn, 2001), we focused effort on ATSIP2 which shared a 76% identity to CMAGAII, shown to be a Raf specific alkaline α-Gal from *Cucumis melo* fruit (Gao and Schaffer, 1999). Using the Sf9 insect cell heterologous expression system, we functionally expressed the *ATSIP2* cDNA demonstrating that it too showed a substrate specificity for Raf, a pH optimum of 8.0 – 8.5 and was completely inhibited by DGJ. Importantly, the recombinant ATSIP2 showed no RafS activity contradicting two recent studies that have reported *ATSIP2* to be a RafS (Nishizawa et al., 2008; Maruyama et al., 2009.

Using the newly identified ATSIP2, we created plant overexpression constructs where *ATSIP2* expression is driven by the CaMV35s promoter (*ATSIP2*::pMDC32) and the water deficit-inducible Rd29A promoter (*ATSIP2*::pCB2010). Due to time constraints this work has not been brought to its conclusion but, we have demonstrated that Arabidopsis transgenic plants (F1) transformed with *ATSIP2*::pMDC32 show a constitutive expression of *ATSIP2* and have higher α-Gal activities, in the leaves, than untransformed plants. We have selected three independent lines that show a high, intermediate and low level of α-Gal activity for further analysis. Transgenic Arabidopsis plants transformed with *ATSIP2*::pCB2010 have been generated but await the preliminary characterisation described above.

A further project, borne from this work, and presently being pursued by Ph.D. candidate Aurelie Egert is to define physiological role/s of ATSIP2 *in vivo*. To date, two promising lines of evidence suggest that ATSIP2 may be involved in distinct physiological processes. Firstly, she has demonstrated that the leaves of T-DNA insertion mutant plants (*atsip2*) contain more Raf 24 h after relief of water deficit than do wild type plants but that there is no difference in leaf Raf concentration between these plants after de-acclimation from 4°C. This suggests that ATSIP2 is involved in the hydrolysis of water deficit-induced Raf, upon the relief of the stress. Secondly, using a 400 bp fragment of genomic DNA upstream of the *ATSIP2* start codon and fusing this to a β-glucoronidase (GUS) gene she has demonstrated that transgenic

Arabidopsis plants show strong GUS activity in sink leaves and roots. This GUS activity correlates to differences in α-Gal activity in these organs, with the highest activities evident in crude extracts from sink leaves and roots. This observation suggests that ATSIP2 may also be involved in phloem unloading as alkaline α-Gals have previously been associated with this function (Keller and Pharr, 1996; Gao and Schaffer, 1999). This is an important finding because Arabidopsis has been reported to transport Suc as a phloem translocate and not any RFOs. Photosynthetic $^{14}C$-pulse chase experiments are currently being conducted on *atsip2* and wild type plants to determine if Raf is transported to some degree in the phloem sap of Arabidopsis.

**REFERENCE LIST**

**Agarwal PK, Agarwal P, Reddy MK, Sopory SK** (2006) Role of DREB transcription factors in abiotic and biotic stress tolerance in plants Plant Cell Reports **25:** 1263-1274

**Albini FM, Murelli C, Finzi PV, Ferrarotti M, Cantoni B, Puliga S, Vazzana C** (1999) Galactinol in the leaves of the resurrection plant *Boea hygroscopica*. Phytochemistry **51:** 499-505

**Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N, Hom E, Karnes M, Mulholland C, Ndubaku R, Schmidt I, Guzman P, Aguilar-Henonin L, Schmid M, Weigel D, Carter DE, Marchand T, Risseeuw E, Brogden D, Zeko A, Crosby WL, Berry CC, Ecker JR** (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science **301:** 653-657

**Altman A** (2003) From plant tissue culture to biotechnology: Scientific revolutions, abiotic stress tolerance, and forestry. In vitro Cellular & Developmental Biology - Plant **39:** 75-84

**Anderson CM, Kohorn BD** (2001) Inactivation of Arabidopsis SIP1 leads to reduced levels of sugars and drought tolerance. Journal of Plant Physiology **158:** 1215-1219

**Arakawa T, Prestrelski SJ, Kenney WC, Carpenter JF** (2001) Factors affecting short-term and long-term stabilities of proteins. Advanced Drug Delivery Reviews **46:** 307-326

**Bachmann M, Keller F** (1995) Metabolism of the raffinose family oligosaccharides in leaves of *Ajuga reptans* L - Intercellular and intracellular compartmentation. Plant Physiology **109:** 991-998

**Bachmann M, Matile P, Keller F** (1994) Metabolism of the raffinose family oligosaccharides in leaves of *Ajuga reptans* L. Cold-acclimation, translocation, and sink to source transition - discovery of chain elongation enzyme. Plant Physiology **105:** 1335-1345

**Barrs HD, Weatherley PE** (1962) A re-examination of the relative turgidity techniques for estimating water deficits in leaves. Australian Journal of Biological Science **15:** 413–428

**Bayer EM, Bottrill AR, Walshaw J, Vigouroux M, Naldrett MJ, Thomas CL, Maule AJ** (2006) Arabidopsis cell wall proteome defined using multidimensional protein identification technology. Proteomics **6:** 301-311

**Bentsink L, Alonso-Blanco C, Vreugdenhil D, Tesnier K, Groot SPC, Koornneef M** (2000) Genetic analysis of seed-soluble oligosaccharides in relation to seed storability of Arabidopsis. Plant Physiology **124:** 1595-1604

**Bernal-Lugo I, Leopold AC** (1995) Seed stability during storage: Raffinose content and seed glassy state. Seed Science Research **5:** 75-80

**Bevan M, Walsh S** (2005) The Arabidopsis genome: A foundation for plant research Genome Research **15:** 1632-1642

**Bianchi G, Gamba A, Limiroli R, Pozzi N, Elster R, Salamini F, Bartels D** (1993) The unusual sugar composition in leaves of the resurrection plant *Myrothamnus flabellifolia*. Physiologia Plantarum **87:** 223-226

**Black M, Corbineau F, Gee H, Come D** (1999) Water content, raffinose, and dehydrins in the iduction of dsiccation torance in immature wheat embryos. Plant Physiology **120:** 463-472

**Blackman SA, Obendorf RL, Leopold AC** (1992) Maturation proteins and sugars in desiccation tolerance of developing soybean seeds. Plant Physiology **100:** 225-230

**Blöchl A, Peterbauer T, Hofmann J, Richter A** (2008) Enzymatic breakdown of raffinose oligosaccharides in pea seeds. Planta **228:** 99-110

**Blöchl A, Peterbauer T, Richter A** (2007) Inhibition of raffinose oligosaccharide breakdown delays germination of pea seeds. Journal of Plant Physiology **164:** 1093-1096

**Bohnert HJ, Ayoubi P, Borchert C, Bressan RA, Burnap RL, Cushman JC, Cushman MA, Deyholos M, Fischer R, Galbraith DW, Hasegawa PM, Jenks M, Kawasaki S, Koiwa H, Kore-eda S, Lee B-H, Michalowski CB, Misawa E, Nomura M, Ozturk N, Postier B, Prade R, Song C-P, Tanaka Y, Wang H, Zhu J-K** (2001) A genomics approach towards salt stress tolerance. Plant Physiology and Biochemistry **39:** 295-311

**Bohnert HJ, Nelson DE, Jensen RG** (1995) Adaptations to environmental stresses. The Plant Cell **7:** 1099-1111

**Bonner CA, Williams DS, Aldrich HC, Jensen RA** (1996) Antagonism by glutamine of toxicity and growth inhibition caused by other amino acids in suspension cultures of *Nicotiana silvestris*. Plant Science **113:** 43-58

**Brenac P, Horbowicz M, Downer SM, Dickerman AM, Smith ME, Obendorf RL** (1997) Raffinose accumulation related to desiccation tolerance during maize (*Zea mays* L) seed development and maturation. Journal of Plant Physiology **150:** 481-488

**Brenac P, Smith ME, Obendorf RL** (1997) Raffinose accumulation in maize embryos in the absence of a fully functional *Vp1* gene product. Planta **203:** 222-228

**Burg MB, Ferraris JD** (2008) Intracellular organic osmolytes: Function and regulation. Journal of Biological Chemistry **283:** 7309-7313

**Cacela C, Hincha DK** (2006) Monosaccharide composition, chain length and linkage type influence the interactions of oligosaccharides with dry phosphatidylcholine membranes. Biochimica et Biophysica Acta **1758:** 680-691

**Caffrey M, Fonseca V, Leopold AC** (1988) Lipid-sugar interactions - relevance to anhydrous biology. Plant Physiology **86:** 754-758

**Cairns AJ, Winters A, Pollock CJ** (1989) Fructan biosynthesis in excised leaves of *Lolium temulentum* L. III. A comparison of the *in vitro* properties of fructosyl transferase activities with the characteristics of *in vivo* fructan accumulation. **112:** 343-352

**Campañá Cué C, Ruiz Salvador A, Aguilera Morales S, Falcon Rodriguez F, Pérez González P** (2001) Raffinose–sucrose crystal interaction modelling. Journal of Crystal Growth **231:** 280-289

**Carlson M** (1999) Glucose repression in yeast. Current Opinion in Microbiology **2:** 202-207

**Carmi N, Zhang G, Petreikov M, Gao Z, Eyal Y, Granot D, Schaffer AA** (2003) Cloning and functional expression of alkaline α-galactosidase from melon fruit: similarity to plant SIP proteins uncovers a novel family of plant glycosyl hydrolases. The Plant Journal **33:** 97-106

**Carpenter JF, Crowe JH** (1989) An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. Biochemistry **28:** 3916-3922

**Carpenter JF, Crowe JH, Arakawa T** (1990) Comparison of solute-induced protein stabilization in aqueous solution and in the frozen and dried states. Journal of Dairy Science **73:** 3627-3636

**Carvalho IS, Chaves M, Pinto-Ricardo C** (2005) Influence of water stress on the chemical composition of seeds of two Lupins (*Lupinus albus* and *Lupinus mutabilis*). Journal of Agronomy and Crop Science **191:** 95-98

**Castrillo M** (1992) Sucrose metabolism in bean plants under water deficit. Journal of Experimental Botany **43:** 1557-1561

**Chen J-Q, Meng X-P, Zhang Y, Xia M, Wang X-P** (2008) Over-expression of *OsDREB* genes lead to enhanced drought tolerance in rice. Biotechnology Letters **30:** 2191-2198

**Chen M, Xu Z, Xia L, Li L, Cheng X, Dong J, Wang Q, Ma Y** (2009) Cold-induced modulation and functional analyses of the DRE-binding transcription factor gene, *GmDREB3*, in soybean (*Glycine max* L.). Journal of Experimental Botany **60:** 121-135

**Chen Y, Burris JS** (1990) Role of carbohydrates in desiccation tolerance and membrane behavior in maturing maize seed. Crop Science **30:** 971-975

**Chinnusamy V, Zhu J, Zhu JK** (2007) Cold stress regulation of gene expression in plants. Trends in Plant Science **12:** 444-451

**Choi DW, Rodriguez EM, Close TJ** (2002) Barley *Cbf3* gene identification, expression pattern, and map location. Plant Physiology **129:** 1781-1787

**Chrost B, Kolukisaoglu U, Schulz B, Krupinska K** (2007) An α-galactosidase with an essential function during leaf development. Planta **225:** 311-320

**Clerkx EJM, El-Lithy ME, Vierling E, Ruys GJ, Blankestijin-De Vries H, Groot SPC, Vreugdenhil D, Koornneef M** (2004) Analysis of natural allelic variation of Arabidopsis seed germination and seed longevity traits between the accessions Landsberg erecta and Shakdara, using a new recombinant inbred line population. Plant Physiology **135:** 432-443

**Clough SJ, Bent AF** (1998) Floral dip: a simplified method for *Agrobacterium* mediated transformation of *Arabidopsis thaliana*. The Plant Journal **16:** 735-743

**Collett H, Butowt R, Smith J, Farrant J, Illing N** (2003) Photosynthetic genes are differentially transcribed during the dehydration-rehydration cycle in the resurrection plant, *Xerophyta humilis*. Journal of Experimental Botany **54:** 2593-2595

**Cook D, Fowler S, Fiehn O, Thomashow MF** (2004) A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America **101:** 15243-15248

**Cunningham SM, Nadeau P, Castonguay Y, Laberge S, Volenec JJ** (2003) Raffinose and stachyose accumulation, galactinol synthase expression, and winter injury of contrasting Alfalfa germplasms. Crop Science **43:** 562-570

**Curtis MD, Grossniklaus U** (2003) A gateway cloning vector set for high-throughput functional analysis of genes *in planta*. Plant Physiology **133:** 462-469

**Cushman JC** (2001) Osmoregulation in plants: Implications for agriculture. American Zoologist **41:** 758-769

**de Vries RP, van den Broeck HC, Dekkers E, Manzanares P, de Graaff LH, Visser J** (1999) Differential expression of three α-galactosidase genes and a

single beta -galactosidase gene from *Aspergillus niger*. Applied and Environmental Microbiology **65:** 2453-2460

**Dèjardin A, Sokolov LN, Kleczkowski LA** (1999) Sugar/osmoticum levels modulate differential abscisic acid-independent expression of two stress-responsive sucrose synthase genes in Arabidopsis. Biochemical Journal **344:** 503-509

**Delauney AJ, Verma DPS** (1993) Proline biosynthesis and osmoregulation in plants. The Plant Journal **4:** 215-223

**Dierking EC, Bilyeu KD** (2009) Raffinose and stachyose metabolism are not required for efficient soybean seed germination. Journal of Plant Physiology **166:** 1329-1335

**Downie B, Bewley JD** (2000) Soluble sugar content of white spruce (*Picea glauca*) seeds during and after germination. Physiologia Plantarum **110:** 1-12

**Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K, Yamaguchi-Shinozaki K** (2003) *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. The Plant Journal **33:** 751-763

**Edwards M, Scott C, Gidley MJ, Reid JSG** (1992) Control of mannose/galactose ratio during galactomannan formation in developing legume seeds. Planta **187:** 67-74

**Ehness R, Ecker M, Godt DE, Roitsch T** (1997) Glucose and stress independently regulate source and sink metabolism and defense mechanisms via signal transduction pathways involving protein phosphorylation. The Plant Cell **9:** 1825-1841

**Erwin A, Galinski AO** (1991) Isolation and structure determination of a novel compatible solute from the moderately halophilic purple sulfur bacterium *Ectothiorhodospira marismortui*. European Journal of Biochemistry **198:** 593-598

**Farrant JM** (2000) A comparison of mechanisms of desiccation tolerance among three angiosperm resurrection plant species. Plant Ecology **151:** 29-39

**Farrant JM** (2007) Mechanisms of desiccation tolerance in angiosperm resurrection plants. *In* A Jenks, AJ Wood, eds, Plant Desiccation Tolerance. CAB International Press, Wallingford, UK, pp 51–90

**Fialho RC, Bücher J** (1995) Changes in levels of foliar carbohydrates and *myo*-inositol before premature leaf senescence of *Populus nigra* induced by a mixture of $O_3$ and $SO_2$. Canadian Journal of Botany **74:** 965–970

**Fowler S, Thomashow MF** (2002) Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. The Plant Cell **14:** 1675-1690

**Foyer CH, Valadier M-H, Migge A, Becker TW** (1998) Drought-induced effects on nitrate reductase activity and mRNA and on the coordination of nitrogen and carbon metabolism in maize leaves. Plant Physiology **117:** 283-292

**Fresneau C, Ghashghaie J, Cornic G** (2007) Drought effect on nitrate reductase and sucrose-phosphate synthase activities in wheat (*Triticum durum* L.): role of leaf internal $CO_2$. Journal of Experimental Botany **58:** 2983-2992

**Gaffney S, Haslam E, Lilley T, Ward T** (1988) Homotactic and heterotactic interactions in aqueous-solutions containing some saccharides - experimental results and an empirical relationship between saccharide solvation and solute–solute interactions. Journal of the Chemical Society, Faraday Transactions **84:** 2545-2552

**Galinski EA** (1993) Compatible solutes of halophilic eubacteria: molecular principles, water-solute interaction, stress protection. Cellular and Molecular Life Sciences **49:** 487-496

**Gancedo JM** (1998) Yeast carbon catabolite repression. Microbiology and Molecular Biology Reviews **62:** 334-+

**Gao Z, Schaffer AA** (1999) A novel alkaline $\alpha$-galactosidase from melon fruit with a substrate preference for raffinose. Plant Physiology **119:** 979-988

**Gaudreault P-R, Webb JA** (1983) Partial purification and properties of an alkaline α-galactosidase from mature leaves of *Cucurbita pepo*. Plant Physiology **71:** 662-668

**Ghasempour HR, Gaff DF, Williams RPW, Gianello RD** (1998) Contents of sugars in leaves of drying desiccation tolerant flowering plants, particularly grasses. Plant Growth Regulation **24:** 185-191

**Gilbert GA, Wilson C, Madore MA** (1997) Root-zone salinity alters raffinose oligosaccharide metabolism and transport in Coleus. Plant Physiology **115:** 1267-1276

**Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF** (2000) Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiology **124:** 1854-1865

**Girousse C, Bournoville R, Bonnemain JL** (1996) Water deficit-induced changes in concentrations in proline and some other amino acids in the phloem sap of Alfalfa. Plant Physiology **111:** 109-113

**Gorecki RJ, Piotrowicz-Cieslak AI, Lahuta LB, Obendorf RL** (1997) Soluble carbohydrates in desiccation tolerance of yellow lupin seeds during maturation and germination. Seed Science Research **7:** 107-116

**Görke B, Stülke J** (2008) Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. Nature Reviews Microbiology **6:** 613-624

**Gösslová M, Svobodová H, Lipavská H, Albrechtová J, Vreugdenhil D** (2001) Comparing carbohydrate status during norway spruce seed development and somatic embryo formation. In Vitro Cellular & Developmental Biology - Plant **37:** 24-28

**Haake V, Cook D, Riechmann JL, Pineda O, Thomashow MF, Zhang JZ** (2002) Transcription factor CBF4 is a regulator of drought adaptation in Arabidopsis. Plant Physiology **130:** 639-648

**Halperin SJ, Koster KL** (2006) Sugar effects on membrane damage during desiccation of pea embryo protoplasts. Journal of Experimental Botany **57:** 2303-2311

**Hamman RA, Jr., Dami IE, Walsh TM, Stushnoff C** (1996) Seasonal carbohydrate changes and cold hardiness of Chardonnay and Riesling grapevines. American Journal of Enology and Viticulture **47:** 31-36

**Han S-E, Park S-R, Kwon H-B, Yi B-Y, Lee G-B, Byun M-O** (2005) Genetic engineering of drought-resistant tobacco plants by introducing the trehalose phosphorylase (TP) gene from *Pleurotus sajor-caju*. Plant Cell, Tissue and Organ Culture **82:** 151-158

**Hannah MA, Wiese D, Freund S, Fiehn O, Heyer AG, Hincha DK** (2006) Natural Genetic Variation of Freezing Tolerance in Arabidopsis. Plant Physiology **142:** 98-112

**Haritatos E, Keller F, Turgeon R** (1996) Raffinose oligosaccharide concentrations measured in individual cell and tissue types in *Cucumis melo* L leaves: Implications for phloem loading. Planta **198:** 614-622

**Haritatos E, Medville R, Turgeon R** (2000) Minor vein structure and sugar transport in *Arabidopsis thaliana*. Planta **211:** 105-111

**Harrison S, Mott E, Parsley K, Aspinall S, Gray J, Cottage A** (2006) A rapid and robust method of identifying transformed *Arabidopsis thaliana* seedlings following floral dip transformation. Plant Methods **2:** 19

**Hatanaka R, Sugawara Y** (2008) 116. Development of desiccation tolerance and vitrification in cultured cells of *Marchantia polymorpha*. Cryobiology **57:** 339-339

**Hayashi AH, Bohnert HJ, Chen THH, Murata N** (1998) Transformation with a gene for choline oxidase enhances the cold tolerance of Arabidopsis during germination and early growth. Plant Cell and Environment **21:** 232-239

**Heck GR, Dorsett C, Ho THD** (1991) Cloning and characterization of a gene Sip1 associated with seed imbibition in barley. Direct submission to gene bank, M77475.

**Hellmann H, Funck D, Rentsch D, Frommer WB** (2000) Hypersensitivity of an Arabidopsis sugar signaling mutant toward exogenous proline application. Plant Physiology **123:** 779-789

**Henrissat B** (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochemical Journal **280:** 309-316

**Henrissat B, Romeu A** (1995) Families, superfamilies and subfamilies of glycosyl hydrolases. Biochemical Journal **311:** 351-352

**Hincha DK, Hagemann M** (2004) Stabilization of model membranes during drying by compatible solutes involved in the stress tolerance of plants and microorganisms. Biochemical Journal **383:** 277-283

**Hincha DK, Zuther E, Heyer AG** (2003) The preservation of liposomes by raffinose family oligosaccharides during drying is mediated by effects on fusion and lipid phase transitions. Biochimica et Biophysica Acta **1612:** 172-177

**Hocking PJ** (1980) The composition of phloem exudate and xylem sap from tree tobacco (*Nicotiana glauca* Grah.). Annals of Botany **45:** 633-643

**Hoekstra FA, Golovina EA, Buitink J** (2001) Mechanisms of plant desiccation tolerance. Trends in Plant Science **6:** 431-438

**Hoekstra FA, Haigh AM, Tetteroo FAA, van Roekel T** (1994) Changes in soluble sugars in relation to desiccation tolerance in cauliflower seeds. Seed Science Research **4:** 143-147

**Holmstrom K-O, Mantyla E, Welin B, Mandal A, Palva ET, Tunnela OE, Londesborough J** (1996) Drought tolerance in tobacco. Nature **379:** 683-684

**Holmstrom K-O, Somersalo S, Mandal A, Palva TE, Welin B** (2000) Improved tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine. Journal of Experimental Botany **51:** 177-185

**Hong Z, Lakkineni K, Zhang Z, Verma DPS** (2000) Removal of feedback inhibition of delta 1-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiology **122:** 1129-1136

**Horbowicz M, Obendorf RL** (1994) Seed desiccation tolerance and storability: dependence on flatulence-producing oligosaccharides and cyclitols - review and survey. Seed Science Research **4:** 385-405

**Huang J, Hirji R, Adam L, Rozwadowski KL, Hammerlindl JK, Keller WA, Selvaraj G** (2000) Genetic engineering of glycinebetaine production toward enhancing stress tolerance in plants: metabolic limitations. Plant Physiology **122:** 747-756

**Huber SC, Huber JL** (1996) Role and regulation of sucrose-phosphate synthase in higher plants. Annual Review of Plant Physiology and Plant Molecular Biology **47:** 431-444

**Hye-Kyoung K, Ryusuke Y, Kazuhiko N** (2005) A Proteomic approach to apoplastic proteins involved in cell wall regeneration in protoplasts of Arabidopsis suspension-cultured cells. Plant and Cell Physiology **46:** 843-857

**Iida K, Seki M, Sakurai T, Satou M, Akiyama K, Toyoda T, Konagaya A, Shinozaki K** (2005) RARTF: Database and tools for complete sets of Arabidopsis transcription factors. DNA Research **12:** 247-256

**Imanishi HT, Suzuki T, Masuda K, Harada T** (1998) Accumulation of raffinose and stachyose in shoot apices of *Lonicera caerulea* L. during cold acclimation. Scientia Horticulturae **72:** 255-263

**Inan-Haab C, Keller F** (2002) Purification and characterization of the raffinose oligosaccharide chain elongation enzyme, galactan:galactan galactosyltransferase (GGT), from *Ajuga reptans* leaves. Plant Physiology **114:** 361-371

**Ingram J, Chandler JW, Gallagher L, Salamini F, Bartels D** (1997) Analysis of cDNA clones encoding sucrose-phosphate synthase in relation to sugar interconversions associated with dehydration in the resurrection plant *Craterostigma plantagineum* Hochst. Plant Physiology **115:** 113-121

**Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF** (1998) Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. Science **280:** 104-106

**Jaglo KR, Kleff S, Amundsen KL, Zhang X, Haake V, Zhang JZ, Deits T, Thomashow MF** (2001) Components of the Arabidopsis C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. Plant Physiology **127:** 910-917

**Jang JC, Leon P, Zhou L, Sheen J** (1997) Hexokinase as a sugar sensor in higher plants. The Plant Cell **9:** 5-19

**Jang JC, Sheen J** (1994) Sugar sensing in higher-plants. The Plant Cell **6:** 1665-1679

**Jouve L, Hoffmann L, Hausman JF** (2004) Polyamine, carbohydrate, and proline content changes during salt stress exposure of Aspen (*Populus tremula* L.): Involvement of oxidation and osmoregulation metabolism. Plant Biology **6:** 74-80

**Kajiwara K, Franks F, Echlin P, Greer AL** (1999) Structural and dynamic properties of crystalline and amorphous phases in raffinose-water mixtures. Pharmaceutical Research **16:** 1441-1448

**Kaplan F, Kopka J, Haskell DW, Zhao W, Schiller KC, Gatzke N, Sung DY, Guy CL** (2004) Exploring the temperature-stress metabolome of Arabidopsis. Plant Physiology **136:** 4159-4168

**Kaplan F, Kopka J, Sung DY, Zhao W, Popp M, Porat R, Guy CL** (2007) Transcript and metabolite profiling during cold acclimation of Arabidopsis reveals an intricate relationship of cold-regulated gene expression with modifications in metabolite content. The Plant Journal **50:** 967-981

**Karner U, Peterbauer T, Raboy V, Jones DA, Hedley CL, Richter A** (2004) myo-Inositol and sucrose concentrations affect the accumulation of raffinose family oligosaccharides in seeds. Journal of Experimental Botany **55:** 1981-1987

**Kasuga M, Miura S, Shinozaki K, Yamaguchi-Shinozaki K** (2004) A combination of the Arabidopsis *DREB1A* gene and stress-inducible rd29A promoter improved drought- and low-temperature stress tolerance in tobacco by gene transfer. Plant and Cell Physiology **45:** 346-350

**Keller F** (1992) Transport of stachyose and sucrose by vacuoles of japanese artichoke (*Stachys sieboldii*) tubers. Plant Physiology **98:** 442-445

**Keller F, Ludlow MM** (1993) Carbohydrate metabolism in drought-stressed leaves of Pigeonpea (*Cajanus cajan*). Journal of Experimental Botany **44:** 1351-1359

**Keller F, Pharr M** (1996) Metabolism of carbohydrates in sinks and sources: galactosyl-sucrose oligosaccharides. *In* E Zamski, AA Schaffer, eds, Photoassimilate Distribution in Plants and Crops: Source-Sink Relationships Marcel Dekker, New York, pp 157-183

**Kim K-N, Guiltinan MJ** (1999) Identification of *cis*-acting elements important for expression of the starch-branching enzyme I gene in maize endosperm. Plant Physiology **121:** 225-236

**King RW, Zeevaart JAD** (1974) Enhancement of phloem exudation from cut petioles by chelating agents. Plant Physiology **53:** 96-103

**Klahn S, Marquardt DM, Rollwitz I, Hagemann M** (2009) Expression of the *ggpPS* gene for glucosylglycerol biosynthesis from *Azotobacter vinelandii* improves the salt tolerance of *Arabidopsis thaliana*. Journal of Experimental Botany **60:**1679-1689

**Klotke J, Kopka J, Gatzke N, Heyer AG** (2004) Impact of soluble sugar concentrations on the acquisition of freezing tolerance in accessions of *Arabidopsis thaliana* with contrasting cold adaptation - evidence for a role of raffinose in cold acclimation. Plant Cell and Environment **27:** 1395-1404

**Koch KE** (1996) Carbohydrate-modulated gene expression in plants. Annual Review of Plant Physiology and Plant Molecular Biology **47:** 509-540

**Koch KE, Nolte KD, Duke ER, Mccarty DR, Avigne WT** (1992) Sugar levels modulate differential expression of maize sucrose synthase genes. The Plant Cell **4:** 59-69

**Konrádová H, Gricová M, Lipavská H** (2003) Cold-induced accumulation of raffinose family oligosaccharides in somatic embryos of Norway spruce (*Picea abies*). In Vitro Cellular & Developmental Biology - Plant **39:** 425-427

**Koster KL, Lei YP, Anderson M, Martin S, Bryant G** (2000) Effects of vitrified and nonvitrified sugars on phosphatidylcholine fluid-to-gel phase transitions. Biophysical Journal **78:** 1932-1946

**Koster KL, Leopold AC** (1988) Sugars and desiccation tolerance in seeds. Plant Physiology **88:** 829-832

**Krapp A, Hofmann B, Schäfer C, Stitt M** (1993) Regulation of the expression of *rbc*S and other photosynthetic genes by carbohydrates: a mechanism for the 'sink regulation' of photosynthesis? The Plant Journal **3:** 817-828

**Kreps JA, Wu Y, Chang H-S, Zhu T, Wang X, Harper JF** (2002) Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. Plant Physiology **130:** 2129-2141

**Kuo TM, VanMiddlesworth JF, Wolf WJ** (1988) Content of raffinose oligosaccharides and sucrose in various plant seeds. Journal of Agricultural and Food Chemisty **36:** 32-36

**Kusano T** (2003) Plant cold hardiness. Gene regulation and genetic engineering. *In* PH Li, ET Palva, eds, Plant cold hardiness. Gene regulation and genetic engineering. Kluwer academic/Plenum publishers, New York, pp 327-328

**Lee B-R, Lan Jin Y, Avice J-C, Cliquet J-B, Ourry A, Kim T-H** (2009) Increased proline loading to phloem and its effects on nitrogen uptake and assimilation in water-stressed white clover *Trifolium repens*. New Phytologist **182:** 654-663

**Lei Z-Y, Zhao P, Cao M-J, Cui R, Chen X, Xiong L-Z, Zhang Q-F, Oliver DJ, Xiang C-B** (2007) High-throughput binary vectors for plant gene function analysis. Journal of Integrative Plant Biology **49:** 556-567

**Leprince O, Hendry GAF, McKersie BD** (1993) The mechanisms of desiccation tolerance in developing seeds. Seed Science Research **3:** 231-246

**Lin T-P, Huang N-H** (1994) The relationship between carbohydrate composition of some tree seeds and their longevity. Journal of Experimental Botany **45:** 1289-1294

**Lippert K, Galinski EA** (1992) Enzyme stabilization be ectoine-type compatible solutes: protection against heating, freezing and drying. Applied Microbiology and Biotechnology **37:** 61-65

**Liu J, Zhu JK** (1997) Proline accumulation and salt stress-induced gene expression in a salt-hypersensitive mutant of Arabidopsis. Plant Physiology **114:** 591-596

**Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K** (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. The Plant Cell **10:** 1391-1406

**Mani S, Van de Cotte B, Van Montagu M, Verbruggen N** (2002) Altered levels of proline dehydrogenase cause hypersensitivity to proline and its analogs in Arabidopsis. Plant Physiology **128:** 73-83

**Mantovani G, Vaccari G, Sgualdino G, Aquilano D, Rubbo M** (1988) Sucrose crystal growth kinetics in the presence of some peculiar impurities. Sugar Journal **50:** 4-6

**Martin DD, Ciulla RA, Roberts MF** (1999) Osmoadaptation in archaea. Applied and Environmental Microbiology **65:** 1815-1825

**Maruyama K, Sakuma Y, Kasuga M, Ito Y, Seki M, M G, Shimada Y, Yoshida S, Shinozaki K, Yamaguchi-Shinozaki K** (2004) Identification of cold-inducible downstream genes of the Arabidopsis DREB1A/CBF3 transcriptional factor using two microarray systems. The Plant Journal **38:** 982-993

**Maruyama K, Takeda M, Kidokoro S, Yamada K, Sakuma Y, Urano K, Fujita M, Yoshiwara K, Matsukura S, Morishita Y, Sasaki R, Suzuki H, Saito K, Shibata D, Shinozaki K, Yamaguchi-Shinozaki K** (2009) Metabolic pathways involved in cold acclimation identified by integrated analysis of metabolites and transcripts regulated by DREB1A and DREB2A. Plant Physiology **150:** 1972-1980

**Mattana M, Biazzi E, Consonni R, Locatelli F, Vannini C, Provera S, Coraggio I** (2005) Overexpression of *Osmyb4* enhances compatible solute accumulation and increases stress tolerance of *Arabidopsis thaliana*. Physiologia plantarum **125:** 212-223

**Matysik J, Bhalu AB, Mohanty P** (2002) Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. Current Science **82:** 525-532

**McCaskill A, Turgeon R** (2007) Phloem loading in *Verbascum phoeniceum* L. depends on the synthesis of raffinose-family oligosaccharides. Proceedings of the National Academy of Sciences of the United States of America **104:** 19619-19624

**McCue KF, Hanson AD** (1990) Drought and salt tolerance: towards understanding and application. Trends in Biotechnology **8:** 358-362

**McNeil SD, Nuccio ML, Hanson AD** (1999) Betaines and related osmoprotectants. targets for metabolic engineering of stress resistance. Plant Physiology **120:** 945-949

**Medina J, Bargues M, Terol J, Perez-Alonso M, Salinas J** (1999) The Arabidopsis CBF gene family is composed of three genes encoding AP2 domain-containing proteins whose expression is regulated by low temperature but not by abscisic acid or dehydration. Plant Physiology **119:** 463-470

**Minic Z, Jouanin L** (2006) Plant glycoside hydrolases involved in cell wall polysaccharide degradation. Plant Physiology and Biochemistry **44:** 435-449

**Moore B, Zhou L, Rolland F, Hall Q, Cheng WH, Liu YX, Hwang I, Jones T, Sheen J** (2003) Role of the Arabidopsis glucose sensor HXK1 in nutrient, light, and hormonal signaling. Science **300:** 332-336

**Müller J, Sprenger N, Bortlik K, Boller T, Wiemken A** (1997) Desiccation increases sucrose levels in *Ramonda* and *Haberlea*, two genera of resurrection plants in the Gesneriaceae. Physiologia Plantarum **100:** 153-158

**Nakashima K, Satoh R, Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K** (1998) A gene encoding proline dehydrogenase is not only induced by proline and hypoosmolarity, but is also developmentally regulated in the reproductive organs of Arabidopsis. Plant Physiology **118:** 1233-1241

**Nakashima K, Shinwari ZK, Sakuma Y, Seki M, Miura S, Shinozaki K, Yamaguchi-Shinozaki K** (2000) Organization and expression of two Arabidopsis DREB2 genes encoding DRE-binding proteins involved in dehydration- and high-salinity-responsive gene expression. Plant Molecular Biology **42:** 657-665

**Nakayama H, Yoshida K, Ono H, Murooka Y, Shinmyo A** (2000) Ectoine, the compatible solute of *Halomonas elongata*, confers hyperosmotic tolerance in cultured tobacco cells. Plant Physiology **122:** 1239-1248

**Nanjo T, Fujita M, Seki M, Kato T, Tabata S, Shinozaki K** (2003) Toxicity of free proline revealed in an Arabidopsis T-DNA-tagged mutant deficient in proline dehydrogenase. Plant and Cell Physiology **44:** 541-548

**Nanjo T, Kobayashi M, Yoshiba Y, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K** (1999) Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. FEBS Letters **461:** 205-210

**Nishizawa-Yokoi A, Yoshida E, Yabuta Y, Shigeoka S** (2009) Analysis of the regulation of target genes by an Arabidopsis heat shock transcription factor, HsfA2. Bioscience, Biotechnology, and Biochemistry **73:** 890-895

**Nishizawa A, Yabuta Y, Shigeoka S** (2008) Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. Plant Physiology **147:** 1251-1263

**Nishizawa A, Yabuta Y, Yoshida E, Maruta T, Yoshimura K, Shigeoka S** (2006) Arabidopsis heat shock transcription factor A2 as a key regulator in response to several types of environmental stress. The Plant Journal **48:** 535-547

**Novillo F, Medina J, Salinas J** (2007) Arabidopsis *CBF1* and *CBF3* have a different function than *CBF2* in cold acclimation and define different gene classes in the CBF regulon. Proceedings of the National Academy of Sciences of the United States of America **104:** 21002-21007

**Obendorf RL** (1998) Oligosaccharides and galactosyl cyclitols in seed desiccation tolerance. Seed Science Research **7:** 63-74

**Ogawa D, Yamaguchi K, Nishiuchi T** (2007) High-level overexpression of the Arabidopsis *HsfA2* gene confers not only increased themotolerance but also salt/osmotic stress tolerance and enhanced callus growth. Journal of Experimental Botany **58:** 3373-3383

**Page-Sharp M, Behm CA, Smith GD** (1999) Involvement of the compatible solutes trehalose and sucrose in the response to salt stress of a cyanobacterial Scytonema species isolated from desert soils. Biochimica et Biophysica Acta **1472:** 519-528

**Panikulangara TJ, Eggers-Schumacher G, Wunderlich M, Stransky H, Schoffl F** (2004) Galactinol synthase1. A novel heat shock factor target gene responsible for heat-induced synthesis of raffinose family oligosaccharides in Arabidopsis. Plant Physiology **136:** 3148-3158

**Papageorgiou GC, Murata N** (1995) The unusually strong stabilizing effects of glycine betaine on the structure and function of the oxygen-evolving photosystem II complex. Photosynthesis Research **44:** 243-252

**Pattanagul W, Madore MA** (1999) Water deficit effects on raffinose family oligosaccharide metabolism in Coleus. Plant Physiology **121:** 987-993

**Patton AJ, Cunningham SM, Volenec JJ, Reicher ZJ** (2007) Differences in freeze tolerance of Zoysiagrasses: II. carbohydrate and proline accumulation. Crop Science **47:** 2170-2181

**Pelleschi S, Rocher JP, Prioul JL** (1997) Effect of water restriction on carbohydrate metabolism and photosynthesis in mature maize leaves. Plant Cell and Environment **20:** 493-503

**Pennycooke JC, Jones ML, Stushnoff C** (2003) Down-regulating $\alpha$-galactosidase enhances freezing tolerance in transgenic petunia. Plant Physiology **133:** 901-909

**Peterbauer T, Mucha J, Mach L, Richter A** (2002) Chain elongation of raffinose in Pea seeds. Isolation, characterisation and molecular cloning of a multifunctional enzyme catalysing the synthesis of stachyose and verbascose. Journal of Biological Chemistry **277:** 194-200

**Peterbauer T, Richter A** (2001) Biochemistry and physiology of raffinose family oligosaccharides and galactosyl cyclitols in seeds. Seed Science Research **11:** 185-197

**Peters S, Keller F** (2009) Frost tolerance in excised leaves of the common bugle (*Ajuga reptans* L.) correlates positively with the concentrations of raffinose family oligosaccharides (RFOs). Plant Cell and Environment **32:** 1099-1107

**Peters S, Mundree SG, Thomson JA, Farrant JM, Keller F** (2007) Protection mechanisms in the resurrection plant *Xerophyta viscosa* (Baker): both sucrose and raffinose family oligosaccharides (RFOs) accumulate in leaves in response to water deficit. Journal of Experimental Botany **58:** 1947-1956

**Prestrelski SJ, Tedeschi N, Arakawa T, Carpenter JF** (1993) Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. Biophysical Journal **65:** 661-671

**Price J, Laxmi A, St. Martin SK, Jang J-C** (2004) Global transcription profiling reveals multiple sugar signal transduction mechanisms in Arabidopsis. The Plant Cell **16:** 2128-2150

**Proctor M, Pence V** (2002) Vegetative tissues: bryophytes, vascular resurrection plants and vegetative propagules *In* M Black, H Pritchard, eds, Desiccation

and survival in plants: drying without dying. CABI Pubishing, Wallingford, UK, pp 207-237

**Ramanjulu S, Bartels D** (2002) Drought- and desiccation-induced modulation of gene expression in plants. Plant Cell and Environment **25:** 141-151

**Rentsch D, Hirner B, Schmelzer E, Frommer WB** (1996) Salt stress-induced proline transporters and salt stress-repressed broad specificity amino acid permeases identified by suppression of a yeast amino acid permease-targeting mutant. The Plant Cell **8:** 1437-1446

**Rentsch D, Schmidt S, Tegeder M** (2007) Transporters for uptake and allocation of organic nitrogen compounds in plants. FEBS letters **581:** 2281-2289

**Riano-Pachon D, Ruzicic S, Dreyer I, Mueller-Roeber B** (2007) PlnTFDB: an integrative plant transcription factor database. BMC Bioinformatics **8:** 42

**Riccardo G, Annick M, Elisabetta G, Jean Pierre G** (1998) Partitioning of photosynthetic carbohydrates in leaves of salt-stressed olive plants. Australian Journal of Plant Physiology **25:** 571-579

**Ritsema T, Smeekens S** (2003) Fructans: beneficial for plants and humans. Current Opinion in Plant Biology **6:** 223-230

**Rohde P, Hincha D, K. , Heyer A, G.** (2004) Heterosis in the freezing tolerance of crosses between two *Arabidopsis thaliana* accessions (Columbia-0 and C24) that show differences in non-acclimated and acclimated freezing tolerance. The Plant Journal **38:** 790-799

**Roosens NHCJ, Thu TT, Iskandar HM, Jacobs M** (1998) Isolation of the ornithine-delta -aminotransferase cDNA and effect of salt stress on its expression in *Arabidopsis thaliana*. Plant Physiology **117:** 263-271

**Salerno GL, Pontis HG** (1989) Raffinose synthesis in *Chlorella vulgaris c*ultures after a cold shock. Plant Physiology **89:** 648-651

**Sanchez DH, Siahpoosh MR, Roessner U, Udvardi M, Kopka J** (2008) Plant metabolomics reveals conserved and divergent metabolic responses to salinity. Physiologia Plantarum **132:** 209-219

**Santarius K** (1973) Protective effect of sugars on chloroplast membranes during temperature and water stress and its relationship to frost, desiccation and heat-resistance. Planta **113:** 105-114

**Santarius KA, Milde H** (1977) Sugar compartmentation in frost-hardy and partially dehardened cabbage leaf cells. Planta **136:** 163-166

**Schwacke R, Grallath S, Breitkreuz KE, Stransky E, Stransky H, Frommer WB, Rentsch D** (1999) LeProT1, a transporter for proline, glycine betaine, and gamma-amino butyric acid in tomato pollen. The Plant Cell **11:** 377-391

**Seki M, Narusaka M, Abe H, Kasuga M, Yamaguchi-Shinozaki K, Carninci P, Hayashizaki Y, Shinozaki K** (2001) Monitoring the expression pattern of 1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. The Plant Cell **13:** 61-72

**Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Taji T, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y, Shinozaki K** (2002) Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. The Plant Journal **31:** 279-292

**Shahba MA, Qian YL, Hughes HG, Koski AJ, Christensen D** (2003) Relationships of soluble carbohydrates and freeze tolerance in saltgrass. Crop Science **43:** 2148-2153

**Sheen J** (1994) Feedback-control of gene-expression. Photosynthesis Research **39:** 427-438

**Shen B, Jensen RG, Bohnert HJ** (1997) Mannitol protects against oxidation by hydroxyl radicals. Plant Physiology **115:** 527-532

**Shinozaki K, Yamaguchi-Shinozaki K** (2007) Gene networks involved in drought stress response and tolerance. Journal of Experimental Botany **58:** 221-227

**Shinwari ZK, Nakashima K, Miura S, Kasuga M, Seki M, Yamaguchi-Shinozaki K, Shinozaki K** (1998) An Arabidopsis gene family encoding DRE/CRT binding oroteins involved in low-temperature-responsive gene expression. Biochemical and Biophysical Research Communications **250:** 161-170

**Sims IM, Cairns AJ, Furneaux RH** (2001) Structure of fructans from excised leaves of New Zealand flax. Phytochemistry **57:** 661-668

**Smart EL, Pharr DM** (1980) Characterization of $\alpha$-galactosidase from cucumber leaves. Plant Physiology **66:** 731-734

**Smeekens S** (2000) Sugar-induced signal transduction in plants. Annual Review of Plant Physiology and Plant Molecular Biology **51:** 49-81

**Somerville C, Koornneef M** (2002) A fortunate choice: the history of Arabidopsis as a model plant. Nature Reviews Genetics **3:** 883-889

**Sprenger N, Keller F** (2000) Allocation of raffinose family oligosaccharides to transport and storage pools in *Ajuga reptans*: the roles of two distinct galactinol synthases. The Plant Journal **21:** 249-258

**Stitt M, Schaewen A, Willmitzer L** (1991) “Sink” regulation of photosynthetic metabolism in transgenic tobacco plants expressing yeast invertase in their

cell wall involves a decrease of the Calvin-cycle enzymes and an increase of glycolytic enzymes. Planta **183:** 40-50

**Sun WQ, Irving TC, Leopold AC** (1994) The role of sugar, vitrification and membrane phase transition in seed desiccation tolerance. Physiologia Plantarum **90:** 621-628

**Székely G, Ábrahám E, Csépl Á, Rigó G, Zsigmond L, Csiszár J, Ayaydin F, Strizhov N, Jásik J, Schmelzer E, Koncz C, Szabados L** (2008) Duplicated *P5CS* genes of Arabidopsis play distinct roles in stress regulation and developmental control of proline biosynthesis. The Plant Journal **53:** 11-28

**Tada N, Sato M, Yamanoi J, Mizorogi T, Kasai K, Ogawa S** (1990) Cryopreservation of mouse spermatozoa in the presence of raffinose and glycerol. Journal of Reproduction and Fertility **89:** 511-516

**Taji T, Ohsumi C, Iuchi S, Seki M, Kasuga M, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K** (2002) Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. The Plant Journal **29:** 417-426

**Taji T, Seki M, Satou M, Sakurai T, Kobayashi M, Ishiyama K, Narusaka Y, Narusaka M, Zhu J-K, Shinozaki K** (2004) Comparative genomics in salt tolerance between Arabidopsis and Arabidopsis-related halophyte Salt Cress using Arabidopsis microarray. Plant Physiology **135:** 1697-1709

**Takagi T, Nakamura M, Hayashi H, Inatsugi R, Yano R, Nishida I** (2003) The leaf-order-dependent enhancement of freezing tolerance in cold-acclimated Arabidopsis rosettes is not correlated with the transcript levels of the cold-inducible transcription factors of CBF/DREB1. Plant and Cell Physiology **44:** 922-931

**Tapernoux-Lüthi EM, Böhm A, Keller F** (2004) Cloning, functional expression, and characterization of the raffinose oligosaccharide chain elongation enzyme, galactan:galactan galactosyltransferase, from common bugle leaves. Plant Physiology **134:** 1377-1387

**Tapernoux-Lüthi EM, Schneider T, Keller F** (2007) The C-terminal sequence from common bugle leaf galactan:galactan galactosyltransferase is a non-sequence-specific vacuolar sorting determinant. FEBS Letters **581:** 1811-1818

**Tarczynski MC, Jensen RG, Bohnert HJ** (1992) Expression of a bacterial mtlD gene in transgenic tobacco leads to production and accumulation of mannitol. Proceedings of the National Academy of Sciences of the United States of America **89:** 2600-2604

**Thomas JC, Sepahi M, Arendall B, J. BH** (1995) Enhancement of seed germination in high salinity by engineering mannitol expression in *Arabidopsis thaliana*. Plant Cell and Environment **18:** 801-806

**Thomas P, Jan M, Ulrike M, Marianne P, Josef G, Andreas R** (1999) Stachyose synthesis in seeds of adzuki bean (*Vigna angularis*): molecular cloning and functional expression of stachyose synthase. The Plant Journal **20:** 509-518

**Thompson JE, Froese CD, Madey E, Smith MD, Yuwen H** (1998) Lipid metabolism during plant senescence. Progress in Lipid Research **37:** 119-141

**Turgeon R, Gowan E** (1990) Phloem loading in *Coleus blumei* in the absence of carrier-mediated uptake of export sugar from the apoplast. Plant Physiology **94:** 1244-1249

**Ueda A, Shi WM, Sanmiya K, Shono M, Takabe T** (2001) Functional analysis of salt-inducible proline transporter of barley roots. Plant and Cell Physiology **42:** 1282-1289

**Ullmann A** (1996) Catabolite repression: A story without end. Research in Microbiology **147:** 455-458

**Valluru R, Lammens W, Claupein W, Van den Ende W** (2008) Freezing tolerance by vesicle-mediated fructan transport. Trends in Plant Science **13:** 409-414

**Van den Ende W, Clerens S, Vergauwen R, Boogaerts D, Le Roy K, Arckens L, Van Laere A** (2006) Cloning and functional analysis of a high DP fructan:fructan 1-fructosyl transferase from *Echinops ritro* (Asteraceae): comparison of the native and recombinant enzymes. Journal of Experimental Botany **57:** 775-789

**Van den Ende W, Van Laere A, Le Roy K, Vergauwen R, Boogaerts D, Figueiredo-Ribeiro RCL, Angela M, de Carvalho M** (2005b) Molecular cloning and characterization of a high DP fructan: fructan 1-fructosyl transferase from *Viguiera discolo*r (Asteraceae) and its heterologous expression in *Pichia pastoris*. Physiologia Plantarum **125:** 419-429

**Verbruggen N, Hermans C** (2008) Proline accumulation in plants: a review. Amino Acids **35:** 753-759

**Verma DPS** (1999) Molecular responses to cold, drought, heat and salt stress in higher plants. *In* K Shinozaki, K Yamaguchi-Shinozaki, eds, Biotechnology Intelligence Unit 1. R.G. Landes Company, Austin, pp 153–168

**Verslues PE, Bray EA** (2006) Role of abscisic acid (ABA) and *Arabidopsis thaliana* ABA-insensitive loci in low water potential-induced ABA and proline accumulation. Journal of Experimental Botany **57:** 201-212

**Vicré M, Farrant JM, Driouich A** (2004) Insights into the cellular mechanisms of desiccation tolerance among angiosperm resurrection plant species. Plant Cell and Environment **27:** 1329-1340

**Vinocur B, Altman A** (2005) Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. Current Opinion in Biotechnology **16:** 123-132

**Wagner W, Keller F, Wiemken A** (1983) Fructan metabolism in cereals: Induction in leaves and compartmentation in protoplasts and vacuoles. Zeitschrift für Pflanzenphysiologie **112:** 357-372

**Wang WX, Vinocur B, Shoseyov O, Altman A** (2001) Biotechnology of plant osmotic stress tolerance physiological and molecular considerations. Acta Hortilcultura **560:** 285-292

**Wanner LA, Junttila O** (1999) Cold-induced freezing tolerance in Arabidopsis. Plant Physiology **120:** 391-400

**Wasilewska A, Vlad F, Sirichandra C, Redko Y, Jammes F, Valon C, Frey NFd, Leung J** (2008) An update on abscisic acid signaling in plants and more. Molecular Plant **1:** 198-217

**Weigel D, Glazebrook J** (2002) Setting up Arabidopsis crosses. *In* D Weigel, J Glazebrook, eds, Arabidopsis: a laboratory manual. CSHL Press, Cold Spring Harbor, New York, pp 41-45

**Werner J, Finkelstein R** (1995) Arabidopsis mutants with reduced response to NaCl and osmotic stress. Physiologia Plantarum **93:** 659-666

**Wiemken V, Ineichen K** (1993) Effect of temperature and photoperiod on the raffinose content of spruce roots. Planta **190:** 387-392

**Wienkoop S, Morgenthal K, Wolschin F, Scholz M, Selbig J, Weckwerth W** (2008) Integration of metabolomic and proteomic phenotypes: Analysis of data covariance dissects starch and RFO metabolism from low and high temperature compensation response in *Arabidopsis thaliana*. Molecular and Cellular Proteomics **7:** 1725-1736

**Xin Z, Browse J** (1998) Eskimo1 mutants of Arabidopsis are constitutively freezing-tolerant. Proceedings of the National Academy of Sciences of the United States of America **95:** 7799-7804

**Yamaguchi-Shinozaki K, Shinozaki K** (1994) A novel *cis*-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. The Plant Cell **6:** 251-264

**Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN** (1982) Living with water-stress: Evolution of osmolyte systems. Science **217:** 1214-1222

**Zhao T-Y, Corum Iii JW, Mullen J, Meeley RB, Helentjaris T, Martin D, Downie B** (2006) An alkaline α-galactosidase transcript is present in maize seeds and cultured embryo cells, and accumulates during stress. Seed Science Research **16:** 107-121

**Zhu C, Cheng C, Liu X, He JY** (2007) Accumulation of soluble sugars related to desiccation tolerance during rice (*Oryza sativa* L) seed development and maturation. Seed Science and Technology **35:** 649-659

**Zhu J, Dong C-H, Zhu J-K** (2007) Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. Current Opinion in Plant Biology **10:** 290-295

**Zhu T, Budworth P, Han B, Brown D, Chang H-S, Zou G, Wang X** (2001) Toward elucidating the global gene expression patternsof developing Arabidopsis: Parallel analysis of 8300 genes by a high-density oligonucleotide probe array. Plant Physiology and Biochemistry **39:** 221-242

**Zimmerman M, Ziegler H** (1975) List of sugars and sugar alcohols in sieve-tube exudates. *In* M Zimmermann, J Milburn, eds, Encyclopedia of Plant Physiology. Springer Verlag, Berlin, pp 480-503

**Zivkovic T, Quartacci MF, Stevanovic B, Marinone F, Navari-Izzo F** (2005) Low-molecular weight substances in the poikilohydric plant *Ramonda serbica* during dehydration and rehydration. Plant Science **168:** 105-111

**Zuther E, Buchel K, Hundertmark M, Stitt M, Hincha DK, Heyer AG** (2004) The role of raffinose in the cold acclimation response of *Arabidopsis thaliana*. FEBS Letters **576:** 169-173

Plant, Cell & Environment

*Plant, Cell and Environment* (2009) **32**, 1099–1107 doi: 10.1111/j.1365-3040.2009.01991.x

# Frost tolerance in excised leaves of the common bugle (*Ajuga reptans* L.) correlates positively with the concentrations of raffinose family oligosaccharides (RFOs)

SHAUN PETERS & FELIX KELLER

*University of Zürich, Institute of Plant Biology, Molecular Plant Physiology, Zollikerstrasse 107, CH-8008 Zürich, Switzerland*

## ABSTRACT

**Mass increases in raffinose family oligosaccharides (RFOs, α1,6-galactosyl extensions of sucrose) are well documented in the generative tissues of many plants upon cold acclimation, and they (i.e. mainly the two shortest RFO members, raffinose and stachyose) have been suggested as frost stress protectants. Our focus here was on the longer RFO members as they commonly occur in the frost-hardy evergreen labiate *Ajuga reptans* in its natural habitat, and accumulate to their highest concentrations in winter when the plant is faced with sub-zero temperatures. We examined the effects of RFO concentration and chain length on frost tolerance using excised leaves which accumulate long-chain RFOs under both cold and warm conditions, thereby uncoupling the acclimation temperature from RFO production. We demonstrated that frost tolerance in excised *A. reptans* leaves correlates positively with long-chain RFO accumulation under both acclimation temperatures. After 24 d post-excision in the warm, the leaves had increased their RFO concentrations (mainly long-chain RFOs) 22-fold to 78 mg $g^{-1}$ fresh weight, and decreased their $EL_{50}$ values (temperature at which 50% leakage occurred) from –10.5 to –24.5 °C, suggesting a protective role for these oligosaccharides in the natural frost tolerance of *A. reptans*.**

*Key-words*: cold acclimation; electrolyte leakage; freezing; water soluble carbohydrates.

## INTRODUCTION

Raffinose family oligosaccharides (RFOs; Suc-$[Gal]_n$, $13 < n \geq 1$) are α1,6-galactosyl extensions of sucrose (Suc) that occur frequently in higher plants. The RFO biosynthetic pathway is initiated with the synthesis of the galactosyl donor galactinol (Gol; 1-O-α-D-galactopyranosyl-L-*myo*-inositol), catalysed by the enzyme galactinol synthase (GolS, EC 2.4.1.123) using UDP-Gal and *myo*-inositol as substrates. Subsequently, the α-galactosyltransferase raffinose (Raf) synthase (RafS, EC 2.4.1.82) transfers a galactosyl moiety from Gol to the $C_6$ position of the glucose (Glc) moiety in Suc, forming an α1,6-galactosidic linkage to yield the trisaccharide Raf. Similarly, stachyose (Sta) synthase (StaS, EC 2.4.1.67) transfers a galactosyl moiety from Gol to the $C_6$ position of the Gal moiety in Raf to yield the tetrasaccharide Sta. In *Ajuga reptans*, a Gol-independent biosynthetic pathway has been reported for higher RFO oligomer biosynthesis (Bachmann & Keller 1995; Haab & Keller 2002; Tapernoux-Lüthi, Böhm & Keller 2004). The enzyme galactan : galactan galactosyltransferase (GGT) utilizes RFOs as galactosyl donors and acceptors during chain elongation, facilitating the synthesis of higher RFO oligomers (up to Suc-$[Gal]_{13}$) in *A. reptans*.

Together with Suc, RFOs have been well characterized as having functional roles in carbon translocation and storage in a number of plant families (Zimmerman & Ziegler 1975; Keller & Pharr 1996). Mass increases in RFOs have also been correlated with the onset of low temperature in a number of plants including alfalfa (Cunningham *et al.* 2003), *Arabidopsis* (Klotke *et al.* 2004), cabbage (Santarius & Milde 1977), salt grasses (Shahba *et al.* 2003), spruce (Wiemken & Ineichen 1993) as well as the photoautotrophic alga *Chlorella vulgaris* (Salerno & Pontis 1989). A recent report has also indicated that, in petunia plants, where an α-galactosidase (α-Gal) had been down-regulated (the first committed step in RFO hydrolysis, catalyzing the cleavage of the terminal α-linked galactose), Raf hyperaccumulation was correlated positively with an increase in frost tolerance (Pennycooke, Jones & Stushnoff 2003). Collectively, such observations firmly place RFOs as having a functional role in low temperature stress. All RFOs share a fundamental characteristic with compatible solutes; as nonreducing sugars, they can accumulate to high intracellular concentrations without affecting metabolic processes. Presumably all RFOs, including higher oligomers, may thus exert protective effects that expand their functional significance (as phloem and storage carbohydrates) to include stress protection.

We have had a long standing research interest in the RFO metabolism of the frost tolerant, evergreen labiate *A. reptans*, and have thus far reported that in this plant: (1) Sta is the main carbon translocate; (2) higher RFO oligomers are the main carbon store (Bachmann, Matile & Keller

*Correspondence: F. Keller. Fax: +41 44 634 8204; e-mail: fkel@botinst.uzh.ch*

1994); and (3) higher RFO oligomers are synthesized by GGT which is targeted to the vacuole via a novel sorting determinant (Bachmann *et al.* 1994; Haab & Keller 2002; Tapernoux-Lüthi *et al.* 2004; Tapernoux-Lüthi, Schneider & Keller 2007). We have also long speculated that the frost tolerant nature of this plant is associated with the high concentrations of RFOs it contains during autumn and winter [up to 200 mg $g^{-1}$ fresh weight (FW) in leaves; Bachmann *et al.* 1994], but have thus far never undertaken to quantify frost resistance with respect to varying RFO concentrations and chain lengths.

The importance of the lowest RFO oligomer, Raf, in the low temperature acclimation and frost tolerance of *Arabidopsis* has recently been challenged (Zuther *et al.* 2004). We were thus interested in analysing the effects of RFO concentration and chain length in the frost tolerance of *A. reptans*. In whole, soil-grown plants, we are only able to induce the accumulation of RFOs through cold acclimation (8/3 °C, 16 h/8 h). Typically, RFOs begin accumulating after 4–6 weeks of acclimation. Given that a number of other low temperature-induced protective pathways come into play during long-term cold acclimation (for review see Chinnusamy, Zhu & Zhu 2007), dissecting a functional role for RFOs in the frost tolerance of *A. reptans* is challenging.

Our aim was, therefore, to employ a system that would effectively uncouple RFO accumulation from low temperature acclimation, thereby allowing us to analyse frost tolerance as a function of RFO concentration. To this end, it has been previously demonstrated that excised leaves of *A. reptans*, placed in water-filled test tubes, rapidly accumulate long-chain RFOs and increase GGT activity in the warm (Haab & Keller 2002; Tapernoux-Lüthi *et al.* 2007). Here, we report on the refinement of the excised leaf system, describing its physiological validation and use as a tool to analyze concentration-dependent effects of RFOs on frost tolerance in *A. reptans*. We demonstrate that: (1) excision does not affect the fundamental carbohydrate physiology of a leaf; and (2) the frost tolerance of excised leaves is improved as RFO concentration increases, suggesting a protective role for RFOs in the natural frost tolerance of *A. reptans*.

## MATERIALS AND METHODS

### Plant material

*Ajuga reptans* plants were grown in a controlled environment chamber (12 h light, 30 $\mu$mol photons $m^{-2}$ $s^{-1}$, 22 °C, 12 h dark, 60% RH) in 6.5 L of Luwasa® hydroculture medium (0.3% v/v, Interhydro AG, Allmendingen, Switzerland) which was continuously aerated using a home aquarium air pump. Growth medium was replaced weekly. Plants were considered experimentation-ready once the root systems had reached approximately 20 cm in length and leaf rosettes were abundant. *Nicotiana benthamiana* plants were propagated from seeds under greenhouse conditions. After 7 d, plants were transplanted: one to a pot and transferred into the growth chamber described above. Plants were considered experimentation-ready after a further 7 d of growth in the chamber.

### Leaf sets

Mature source leaves of similar size were excised from rosettes with a scalpel and the ends of the petioles subsequently re-cut under water. Petioles were immersed in a test tube, filled with water and capped with a perforated black plastic cap that allowed the leaf petiole through. Leaf sets (six leaves per set) were then placed in a test tube rack covered with black PVC sheeting, exposing only the leaf to light and maintained in the controlled environment chamber described above. One leaf set was subsequently sampled every 6 d over a period of 30 d and processed for carbohydrate and enzyme extractions, as well as frost tolerance experiments. *N. benthamiana* leaves were treated in the same manner, except that leaf sets were sampled at 6, 9 and 12 d.

### Water soluble carbohydrate (WSC) extraction

WSCs were extracted using an ethanol series, as previously described (Peters *et al.* 2007) with modifications. Two leaf discs (6 mm ∅) were punched out of each leaf in a set, weighed, flash-frozen in liquid $N_2$ and macerated using a plastic pestle in a 1.5 mL Eppendorf tube. WSCs were extracted twice (per step) in a three-step sequential process, using 100 $\mu$L 10 mg $g^{-1}$ FW of 80% EtOH, 50% EtOH and $dH_2O$. Extractions were conducted at 85 °C for 10 min and the tubes centrifuged at 12 000 *g* (5 min, 4 °C). Supernatants were removed to a separate Eppendorf tube before the next extraction in the sequence. The supernatants of all extractions were pooled and concentrated in a vacuum concentrator centrifuge. Extracts were then re-suspended in 100 $\mu$L of $dH_2O$. Aliquots (50 $\mu$L) were de-ionized and de-phenolized as described below, and analysed by high-performance liquid chromatography with pulsed amperometric detection (HPLC-PAD).

### Enzyme extractions and activity assays

#### *Plant crude extracts*

Freshly harvested leaf material (200 mg) was ground in 500 $\mu$L of chilled extraction buffer A for alkaline enzyme extracts [50 mM Hepes/KOH pH 7.5, 5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 20 mM DTT, 0.1% (v/v) Triton X-100, 1 mM benzamidine, 1 mM PMSF, 50 mM Na ascorbate, 2% (w/v) PVP] or extraction buffer B for acidic extracts [50 mM trisodium citrate, 20 mM DTT, 5 mM $MgCl_2$, 2% PEG 6000 (w/v), 2% PVP K30 (w/v) pH 5.0]. Samples were transferred into 2 mL Eppendorf tubes and centrifuged at 12 000 *g* (10 min, 4 °C). A 150 $\mu$L aliquot of supernatant was desalted by gel filtration at 1400 *g* (2 min, 4 °C) through 5 mL Sephadex G-25 columns (fine, final bed volume of 3 mL). Columns were pre-equilibrated with an alkaline assay buffer (50 mM Hepes/

KOH pH 7.5, 2 mM $MnCl_2$, 10 mM DTT) or an acidic assay buffer (McIlvaine Buffer pH 5.0). Pre-equilibration was performed twice by centrifugation at 1400 *g* (2 min, 4 °C), each with 2 mL of assay buffer. Aliquots (20 $\mu$L) of desalted extract were assayed for respective enzyme activities in a final volume of 40 $\mu$L assay buffer containing 100 mM Suc and 10 mM Gol for RafS, 50 mM Raf and 5 mM Gol for StaS, 100 mM Raf for GGT, and both acidic and alkaline $\alpha$-Gals. All assays were conducted for 1 h at 30 °C and stopped by flash-freezing the tubes in liquid $N_2$, and subsequently boiling for 5 min. Samples were de-ionized, de-phenolized and analysed by HPLC-PAD.

### *Desalting of extracts*

Desalting of carbohydrate and enzyme assay samples to remove phenolics and ions was conducted by centrifuge-rinsing of the samples through pre-rinsed 1 mL Mobicol spin columns (MoBiTec, Göttingen, Germany), as previously described (Peters *et al.* 2007) with minor modifications. Aliquots of ethanol extracts (75 $\mu$L) were desalted and centrifuge-rinsed twice, each with 175 $\mu$L of $dH_2O$. Desalted samples were then concentrated in a vacuum concentrator centrifuge and re-suspended in 100 $\mu$L of $dH_2O$ prior to HPLC-PAD. All 40 $\mu$L of enzyme assay reactions were desalted and centrifuge-rinsed once with 100 $\mu$L of $dH_2O$.

### *HPLC-PAD analysis*

Desalted carbohydrate extracts and enzyme assay reactions were analysed and quantified by HPLC-PAD as described in Peters *et al.* (2007). Briefly, two chromatographic systems using either a $Ca^{2+}/Na^{+}$-moderated ion partitioning carbohydrate column (Benson BC100, BC200 columns, 7.8 × 300 mm; Benson Polymeric, Reno, Nevada, USA), or an anion exchange CarboPac PA1 column (4 × 250 mm; Dionex, Sunnyvale, CA, USA) were used to separate carbohydrates. Quantification was done using the Chromeleon v6.4 software package against a series of 5 nmol of standard sugars, the concentration of which corresponded to the linear response range of both chromatographic systems. In the absence of commercially available higher RFO oligomer standards, long-chain RFOs (DP, degree of polimerization, > 5) were quantified against a verbascose (Ver; DP5) standard and are presented as Ver equivalents.

### *Pulse-chase experiments*

Six excised leaves were maintained in the controlled environment chamber described above for 6 d, then placed in a glass thin layer chromatography chamber. Two 1.5 mL Eppendorf tubes were scotch taped to the sidewalls. To introduce $^{14}CO_2$ into the system, $NaH^{14}CO_3$ (Hartmann Analytic, Braunschweig, Germany) was added to the Eppendorf tubes (0.925 MBq $g^{-1}$ FW, specific activity 2183 MBq $mmol^{-1}$). Lactic acid was added to each of the tubes and the chromatography tank was sealed with a glass lid lined with silicone grease. Leaves were subjected to strong illumination (460 $\mu$mol photons $m^{-2}$ $s^{-1}$) for 15 min and chased with $^{12}CO_2$ for 2 h, on the benchtop, without additional illumination. Leaves were then processed either for carbohydrate extractions described above or phloem exudation.

### *Phloem exudations*

Phloem exudations were based on the method previously described (King & Zeevaart 1974; Bachmann *et al.* 1994), with modifications. After pulsing leaves with $^{14}CO_2$, petiole ends were re-cut under exudation buffer (5 mM EDTA, 5 mM $KHPO_4$ pH 7.0) and placed in an Eppendorf tube filled with exudation buffer. Exudations were allowed to proceed for 2–4 h in a sealed, humidified chromatography chamber. Subsequently, the samples were analysed for $^{14}C$-carbohydrates by separation on a BC100 column coupled to a FLO-ONE radio chromatography detector (Model A-525X, Packard, Zürich, Switzerland).

### *Frost tolerance experiments*

Leaf sets were harvested at various post-excision times (0, 6, 12, 18 and 24 d) and subjected to freezing temperatures of –5, –10, –15 and –20 °C in a programmable freezing chamber (RUMED, Blanc-Labo S.A., Lonay, Switzerland). Prior to being placed in the chamber, leaves were transferred into plastic bags containing a few ice chips and the end of the bags tied off loosely with string. For each freezing temperature, the chambers were programmed to decrease (from –1 °C) by –1 °C $h^{-1}$ and hold at the designated temperature for 3 h before increasing by +1 °C $h^{-1}$ until the hold temperature of –1 °C was reached. Subsequently, the plastic bags were placed on ice to thaw. Two leaf discs (6 mm ∅) were punched out of each leaf. Discs were placed immediately into the wells of a six-well microtiter plate and incubated at room temperature for 1 h with agitation. Each well contained 1.5 mL of de-ionized water. The conductivity was measured for each well using a conductivity meter (Model 712, Metrohm, Zofingen, Switzerland) and this represented the initial leakage ($L_i$). Leaf discs were then flash-frozen in liquid $N_2$, quickly re-immersed in the same well they had originally been in and incubated for 1 h as described above. Conductivity was measured for each well and this represented the total leakage ($L_t$). Electrolyte leakage was expressed as a relative percentage of total leakage [($L_i$ $L_t^{-1}$) ∗ 100]. The temperature at which 50% relative leakage occurred from these data was defined as the $EL_{50}$. For each series of inductions, $EL_{50}$ values were obtained by plotting non-linear (sigmoidal) regression curves through scatter plots of the relative leakage data obtained for each freezing temperature.

## RESULTS

### The leaves of *A. reptans* plants exhibit two distinct RFO states with differing frost tolerance

HPLC-PAD analysis of total leaf WSCs indicated that warm-grown plants (22 °C) accumulated proportionally

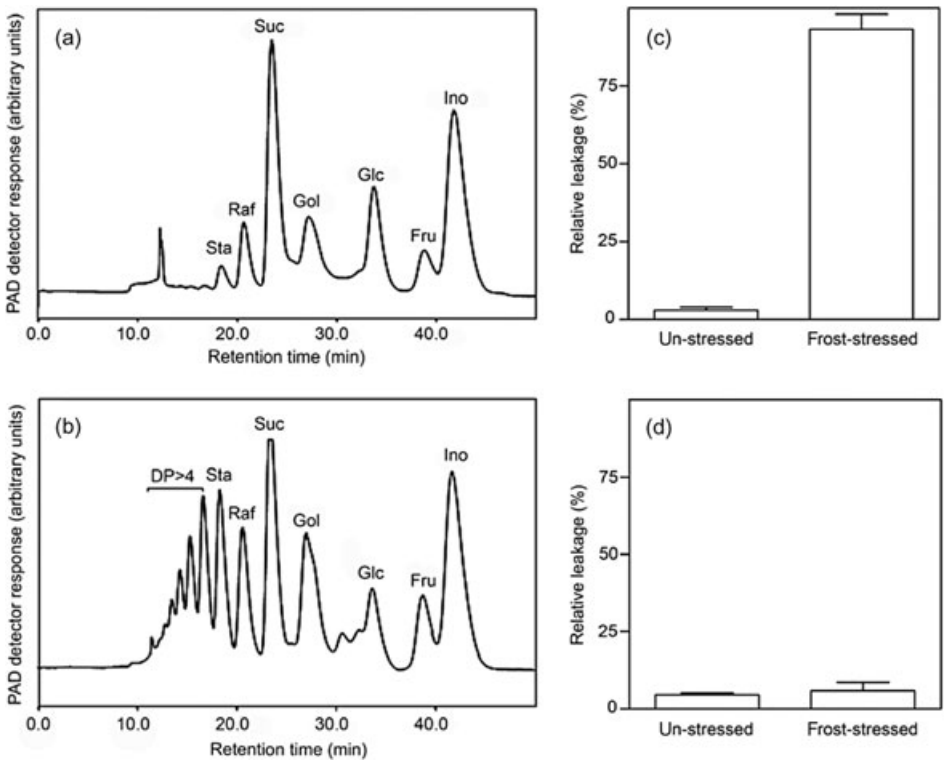


**Figure 1.** Water soluble carbohydrates (WSCs) and frost tolerance of leaves from warm-grown (22 °C; a, c) and cold-grown (8/3 °C, day/night; b, d) *Ajuga reptans* plants. Graphs a and b represent the WSC profiles, while graphs c and d represent the relative electrolyte leakage from leaves after exposure to a freezing stress of –20 °C. Data points represent the mean ± SE of six replicates. Samples were analysed by high-performance liquid chromatography using the BC200 column. Fru, fructose; Glc, glucose; Gol, galactinol; Ino, *myo*-inositol. PAD, pulsed amperometric detection; Raf, raffinose; Sta, stachyose; Suc, sucrose.

lower amounts of total RFOs, with Sta (DP4) being the highest RFO oligomer detectable (Fig. 1a). In cold-grown plants (8 °C/3 °C, day/night), RFOs were proportionally higher and were detectable as higher oligomers up to about DP9 (Fig. 1b). When leaves from both of these plants were exposed to a freezing stress of –20 °C, relative electrolyte leakage differed greatly. In the leaves of warm-grown plants, it was >90%, although it was 6% in the leaves of cold-grown plants, comparable to the relative leakage of un-stressed, warm-grown control leaves (Fig. 1c & d, respectively).

## Excised leaves produce mainly $^{14}$C-Suc as a photosynthate and export mainly $^{14}$C-Sta as a phloem translocate in $^{14}CO_2$ photosynthetic pulse-chase experiments

Photosynthetic pulse-chase experiments using $^{14}CO_2$ were conducted with excised leaves at 6, 12 and 24 d after excision (DAE). Radio-HPLC analyses of the leaf WSCs indicated that the major proportion of photosynthetically fixed carbon at all time points consistently occurred as $^{14}$C-Suc, along with $^{14}$C-Raf, $^{14}$C-Sta, $^{14}$C-Gol, $^{14}$C-Glc and $^{14}$C-Fru (fructose) (Fig. 2a, representative, 12 DAE). Radio-HPLC analyses of the EDTA phloem exudates from these leaves indicated that the predominant $^{14}$C-WSC in the phloem sap was $^{14}$C-Sta (Fig. 2b, representative, 12 DAE).

## Warm-incubated (22 °C) excised leaves accumulate RFOs independent of cold acclimation

The changes in WSC concentrations were analysed in warm-incubated excised leaves at 6 d intervals over a period of 30 d. The total RFO concentration increased nearly 22-fold to 82 mg g$^{-1}$ FW (Fig. 3). Higher RFO oligomers (DP > 4) accounted for the largest portion, showing a 74-fold increase from 0.6 to 57 mg g$^{-1}$ FW after 30 d. This correlated positively with an eightfold increase in GGT activity from 5.6 to 46.4 nkat g$^{-1}$ FW. Sta increased 7.7-fold from 1.8 to 13.7 mg g$^{-1}$ FW. Raf increased sevenfold over the same period from 1.1 to 8 mg g$^{-1}$ FW. A clear positive correlation between Raf and RafS activity was not evident.

Suc concentration was nearly constant, showing a relatively minor 1.4-fold increase from 3.2 to 4.5 mg g$^{-1}$ FW (Fig. 3). Activities for RFO hydrolytic enzymes indicated that alkaline $\alpha$-Gal activity did not change over the 30 d period. However a pronounced increase in acidic $\alpha$-Gal activity occurred between 18 and 30 DAE (Fig. 3). Together with control leaves, we chose leaves at 12 and 24 DAE to represent low, intermediate and high RFO states,

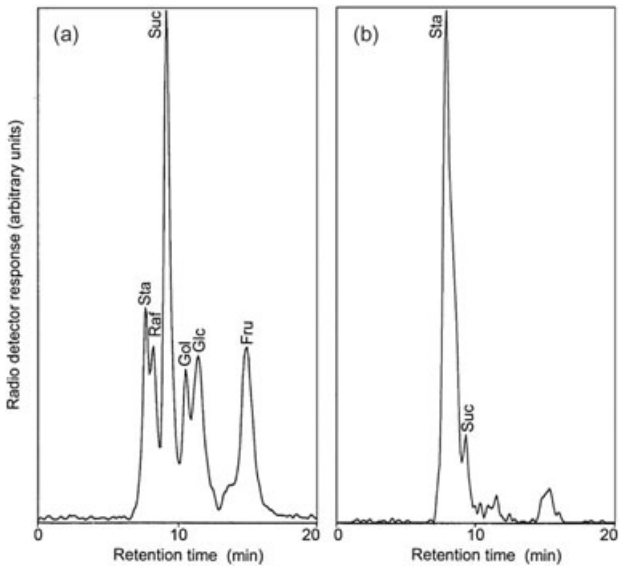


**Figure 2.** Representative radio high-performance liquid chromatography of $^{14}$C-WSCs $^{14}CO_2$ photosynthetic pulse-chase experiments conducted on warm-incubated (22 °C) *Ajuga reptans* excised leaves (12 DAE), and analysed for carbohydrates (a) and phloem exudation (b). Samples were analysed using the BC100 column. DAE, days after excision; Fru, fructose; Glc, glucose; Gol, galactinol; Raf, raffinose; Sta, stachyose; Suc, sucrose; WSC, water soluble carbohydrates.

respectively, to be subjected to frost tolerance experiments as described.

### Electrolyte leakage and $EL_{50}$ are lower in leaves with high RFO concentration

Excised leaves from 12 and 24 DAE were exposed to freezing temperatures of –5 to –20 °C. Leaves at 24 DAE displayed the lowest relative electrolyte leakage over all temperatures tested (Fig. 4). Similarly, leaves at 12 DAE showed an intermediate relative leakage between that of the control and 24 DAE. Temperatures of –15 and –20 °C resulted in similar average relative leakage of 75% for controls, 60% for 12 DAE and 45% for 24 DAE, suggesting that –15 °C is the upper limit of tolerance for leaves at these RFO states.

Excised leaves with a low RFO concentration (controls) had an $EL_{50}$ of about –10.5 °C (Fig. 5). As RFO concentrations in excised leaves increased, the $EL_{50}$ temperature decreased further with leaves showing an $EL_{50}$ of –16.0 °C at 12 DAE and –24.5 °C at 24 DAE (Fig. 5). The strongest correlation between $EL_{50}$ and RFO concentration was evident for higher RFO oligomers (>Sta). Excised leaves of *N. benthamiana* had no detectable RFOs over 12 DAE when incubated under both cold and warm conditions. The frost tolerance of leaves incubated in the warm did not improve over this period, with leaves 12 DAE showing a slightly higher (75%) relative electrolyte leakage than leaves 6 DAE, when challenged by freezing at –5 °C (50%, Fig. 4b). Excised *N. benthamiana* leaves incubated in the cold showed a marked improvement in frost tolerance 6 DAE, compared with control leaves with relative electrolyte leakage of about 10% at –5 °C but this tolerance did not improve over 12 DAE.

## DISCUSSION

### *A. reptans* plants exist in two distinct physiological states that differ in frost tolerance

*Ajuga reptans* exists naturally in two distinct physiological states relative to RFO pools. It has been reported that during spring and summer in Zürich (April to August), RFO concentrations in this plant are relatively low, attaining concentrations of 74 and 42 mg $g^{-1}$ FW in aerial parts and roots, respectively (Bachmann *et al.* 1994). In the transition from late summer to autumn (late August), dramatic mass increases in RFOs occur, attaining maximum concentrations of 200 and 110 mg $g^{-1}$ FW in aerial parts and roots, respectively. Presumably, this represents primarily the storage function of the total RFO pool, as concentration decreases occur in aerial parts and roots throughout late winter to summer (February to June), suggesting a remobilization of the storage RFO pool towards growth in this perennial plant.

Our work on the RFO physiology of *A. reptans* has long pointed towards a second function of these oligosaccharides, i.e. in frost tolerance. The highest RFO concentrations coincide with the winter season, when plants are exposed to the most extreme of low temperatures in Zürich (average day temperature of between 0 and –5 °C, http://www.meteoschweiz.ch). Given that RFO mass increases up to at least Sta have been observed to occur in plants exposed to low temperature (Cunningham *et al.* 2003; Shahba *et al.* 2003; Klotke *et al.* 2004), we quantified the frost tolerance in leaves of cold-grown (8/3 °C, day/night) and warm-grown (22 °C) *A. reptans* plants.

The leaves of warm-grown plants accumulated proportionally lower amounts of RFOs, with Sta being the highest oligomer observed. The leaves of cold-grown plants, however, accumulated proportionally higher amounts of RFOs, with RFOs accumulating to higher oligomers (up to Suc-$[Gal]_7$, Fig. 1). Importantly, after a –20 °C freezing stress, the leaves of cold-grown plants displayed a low relative electrolyte leakage that was comparable to un-stressed control leaves. Leaves of warm-grown plants showed high relative leakage values of >90%, demonstrating that leaves from cold-grown plants are more frost tolerant. To further dissect a role for RFOs in this tolerance, we refined a previously described excised leaf system that would effectively uncouple RFO accumulation from cold acclimation (Haab & Keller 2002).

### Long-term cold acclimation of excised leaves results in additive frost tolerance

Traditionally, we have induced RFO accumulation by exposing whole plants to a low-temperature growth regime

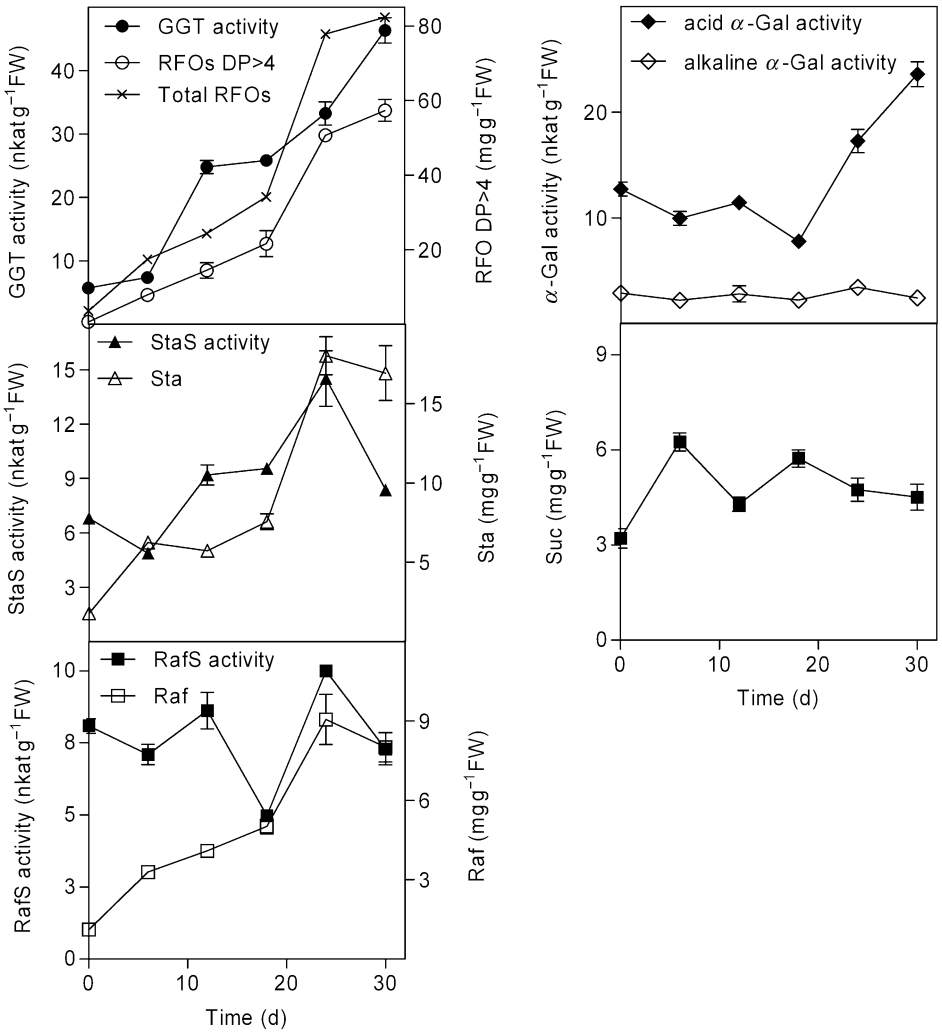


**Figure 3.** WSC and enzyme activity changes in warm-incubated (22 °C) *Ajuga reptans* excised leaves over a 30 d period. Data points represent the mean ± SE of six replicates. Enzyme assay samples were analysed by high-performance liquid chromatography using the BC100 column, while water soluble carbohydrates samples were analysed using both the PA1 and the BC100 columns. α-Gal, α-galactosidase; GGT, galactan : galactan galactosyltransferase; Raf, raffinose; RafS, raffinose synthase; Sta, stachyose; StaS, stachyose synthase; Suc, sucrose.

(8–10/3 °C, day/night) to mimic the transition from late summer to autumn, when *A. reptans* naturally accumulates large amounts of RFOs (Bachmann *et al.* 1994). However, any attempt to analyse a functional role for RFOs in frost tolerance under this regime is met with the problem of the plethora of regulons induced during low temperature (Chinnusamy *et al.* 2007). Cold-incubated excised *A. reptans* leaves accumulate slightly less soluble sugars, in totality, than warm-incubated ones (Haab & Keller 2002). However at 18 DAE, cold-incubated excised leaves are more resistant to –20 °C than warm-incubated ones (Fig. 6). A traditional interpretation of such data would immediately discount a role for soluble sugars in frost tolerance. We, however, maintain that under conditions of long-term cold acclimation, additive protective effects can mask the functional role/s of single components such as RFOs. To use an excised leaf system to examine the role of RFOs in the frost tolerance of *A. reptans*, we first examined the effects of excision on carbohydrate metabolism in leaves and compared these data to that of whole plants.

## Excised leaves produce $^{14}$C-Suc and export $^{14}$C-Sta

The physiological validity of using an excised leaf system to uncouple RFO accumulation from cold acclimation in *A. reptans* was tested by inducing RFO accumulation over a period of 30 d under ambient growth conditions (22 °C). Using classical photosynthetic $^{14}CO_2$ pulse-chase experiments, we demonstrated that at 6, 12 and 24 DAE, single excised leaves operate within normal photosynthetic parameters, producing $^{14}$C-Suc as the major photosynthate, aside from about 10% each of $^{14}$C-Gol, $^{14}$C-Raf and $^{14}$C-Sta (Fig. 2a, representative, 12 DAE). This $^{14}$C-photosynthate

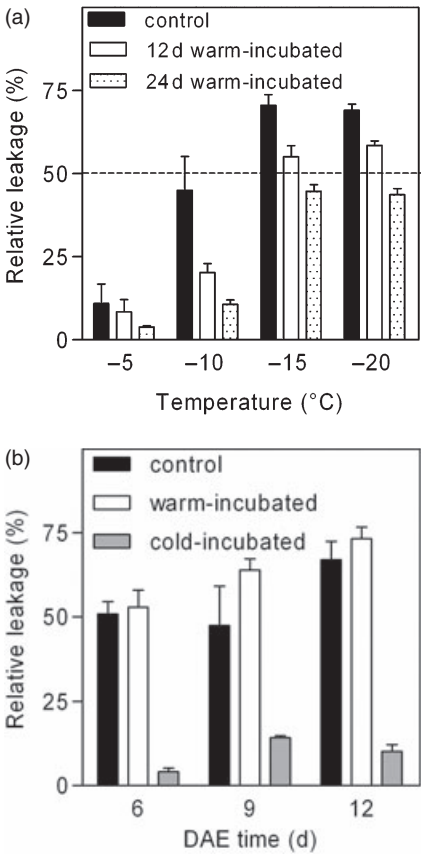


**Figure 4.** Relative electrolyte leakage of (a) warm-incubated (22 °C) *Ajuga reptans* excised leaves exposed to freezing temperatures of between –5 and –20 °C, and (b) warm-incubated (22 °C) and cold-incubated (8/3 °C, day/night) *Nicotiana benthamiana* excised leaves exposed to –5 °C. Control leaves were excised directly from warm-grown plants and subjected to freezing. Data points represent the mean ± SE of six replicates. DAE, days after excision.

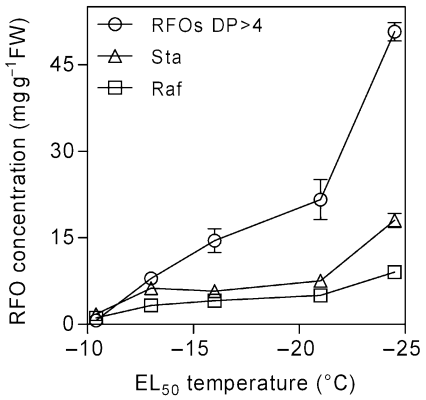


**Figure 5.** $EL_{50}$ values plotted as a function of raffinose family oligosaccharides (RFO) concentration increases in warm-incubated (22 °C) *Ajuga reptans* excised leaves over a 30 d period. Data points represent the mean ± SE of six replicates at 6 d intervals. DP, degree of polymerization; FW, fresh weight; Raf, raffinose; Sta, stachyose.

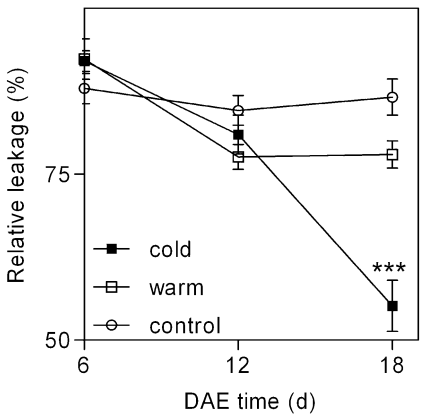


**Figure 6.** Relative electrolyte leakage of warm-incubated (22 °C) and cold-incubated (8/3 °C, day/night) *Ajuga reptans* excised leaves exposed to a freezing stress of –20 °C. Control leaves were excised directly from in warm-grown (22 °C) plants and subjected to freezing at –20 °C. Data points represent the mean ± SE of six replicates. Statistical significance of a two-tailed *t*-test is represented by stars ($P < 0.05$). DAE, days after excision.

pattern is quite similar to that obtained earlier using leaf discs and strips freshly isolated from *A. reptans* leaves (Bachmann & Keller 1995). Furthermore, analysis of phloem exudates after pulsing excised leaves with $^{14}CO_2$ in the light indicated that the major WSC in the phloem is $^{14}$C-Sta (Fig. 2b, representative, 12 DAE), as we have previously reported using *A. reptans* whole plants (Bachmann *et al.* 1994). By removing a leaf from the plant, the major physiological change that we propose to occur is the channelling of newly fixed carbon to the mesophyll vacuolar storage pool (Bachmann & Keller 1995), without any measurable effect on (1) photosynthesis and (2) the ability of the leaf to re-establish phloem transport through an incubation of the petiole excision zone in 5 mM EDTA. Obviously, the possibility of additional effects of leaf excision on frost tolerance cannot be totally excluded but might be minimal.

## Excised leaves accumulate RFOs under warm growth conditions

As reported previously, RFOs increase in warm-incubated excised leaves (Haab & Keller 2002). We have also measured GGT, RafS and StaS activities, showing that GGT activity increases over 30 d correlate with the large increases in higher RFO oligomer concentrations (Fig. 3). Activity measurements for $\alpha$-Gals, catalysing the first committed RFO degradation step, indicated that while alkaline $\alpha$-Gal activity remained unchanged and low over a 30 d period, a very pronounced increase in acid $\alpha$-Gal activity was observed after 18 DAE (Fig. 3). This could be correlated with the emergence of root buds at the excision zone of petioles, observed after 12–14 DAE. Assuming that this temporal window represents the point at which an excised leaf begins to reactivate its source activity, we postulate that

this developmental switch triggers re-mobilization of the RFOs in the mesophyll vacuoles and phloem export of carbon to fuel new root growth.

We have previously shown the phloem loading mechanism in *A. reptans* to be in accordance with the polymer trap model of symplastic loading (Turgeon & Gowan 1990; Bachmann & Keller 1995). This would imply that higher RFO oligomers in the vacuoles are re-mobilized by hydrolysis to Suc, which then diffuses into intermediary cells to be immediately re-synthesized to Raf and Sta, and exported via the phloem from the sieve elements. Unsurprisingly, Suc concentration remained relatively constant compared with RFOs, highlighting the fundamental physiology of *A. reptans*, where post-photosynthetic carbohydrate metabolism is primed to favour RFO biosynthesis.

## Frost tolerance in excised leaves correlates positively with RFO concentration

Most importantly, we have obtained a clear positive correlation between RFO concentrations and the frost tolerance of excised leaves in the absence of cold acclimation. All control leaves had the lowest total RFO concentration (3.6 mg $g^{-1}$ FW) and were tolerant to –5 °C, as evidenced by low relative electrolyte leakage (Fig. 4).

Total leaf RFO concentration increased nearly 22-fold in excised leaves in the warm, 24 DAE, and leaves at 12 and 24 DAE were much more tolerant to freezing temperatures of up to –20 °C than control leaves. The total RFO increase correlated positively with a shift in the $EL_{50}$ value from about –10.5 °C (control) to –24.5 °C (Fig. 5), showing for the first time quantitatively that, in *A. reptans* leaves, RFOs may improve frost tolerance irrespective of cold acclimation. The best correlation to the shift in $EL_{50}$ values was evident between concentration increases in higher RFO oligomers, which accounted for 65% of the total RFO increase. As a control, we also examined the effects of leaf excision on frost tolerance, using excised leaves of *N. benthamiana*. Our HPLC-PAD analysis indicated that no RFOs occurred in either warm- or cold-incubated excised leaves up to 12 DAE, in accordance with previous reports for tobacco (Haritatos, Keller & Turgeon 1996). Importantly, excised *N. benthamiana* leaves incubated in the warm did not show any improvement in frost tolerance (Fig. 4b), despite a 3.5-fold Suc increase to about 418 mg $g^{-1}$ FW 12 DAE. A similar Suc increase (3.4-fold) was evident in cold-incubated excised *N. benthamiana* leaves to about 384 mg $g^{-1}$ FW. However, frost tolerance was markedly improved in cold-incubated leaves 6 DAE, suggesting that other factors apart from Suc may be important in cold acclimation-induced frost protection of *N. benthamiana* excised leaves.

One of the *in vitro* experimentally demonstrated functions of the lowest RFO oligomer, Raf, is its ability to hydrogen bond to biomolecules more effectively than either Suc or trehalose, considered to be the most abundant stress protective sugars in nature (Gaffney *et al.* 1988). This direct hydrogen bonding between sugars and biomolecules has been demonstrated to be imperative, even in low concentrations, in the stabilization of proteins, membranes and whole cells under conditions of freezing and dehydration (Carpenter & Crowe 1989; Prestrelski *et al.* 1993; Koster *et al.* 2000; Arakawa *et al.* 2001; Hincha, Zuther & Heyer 2003; Hatanaka & Sugawara 2008).

The protective efficacy of higher RFO oligomers in frost tolerance cannot be precluded on the basis of the compatible solute nature of these non-reducing sugars. Indeed, there is *in planta* evidence that together with Raf, Sta may play an important role in cold hardiness of *Loniceria caerula* L. shoot apices (Imanishi *et al.* 1998), alfalfa roots (Cunningham *et al.* 2003), as well as Chardonnay and Riesling grapevines (Hamman *et al.* 1996). From *in vitro* studies, it has been reported that commercially available RFO oligomers (up to Ver) offer increasing protection to artificial liposomes, during drying, with increasing chain length (Hincha *et al.* 2003). Similar *in vitro* observations were made for inulin-type fructan oligomers ($\beta$2,1-fructosyl extensions of Suc, Suc-$[Fru]_n$) where lyoprotection was positively correlated with increasing chain length (Cacela & Hincha 2006). Our data has provided convincing *in planta* evidence that frost tolerance in *A. reptans* leaves may depend on RFO concentration, as the excised leaf system uncouples RFO accumulation from cold acclimation. Furthermore, the best positive correlation to increases in $EL_{50}$ were evident for higher RFO oligomers (>Ver), suggesting that they may be important contributors to frost tolerance in this plant. Because these higher RFO oligomers are synthesized and stored in the large central vacuole of mesophyll cells (Bachmann & Keller 1995), the question arises if they may exert their protective role also outside the vacuole, e.g. in the plasma membrane. To do so, they would need to reach the plasma membrane, but no such mechanism has been described for RFOs nor has their putative apoplastic location been determined. The recently proposed model for vesicle-mediated transport of fructans from the vacuole to the apoplast to assist in stabilizing the plasma membrane (Valluru *et al.* 2008) might also be applicable to RFOs and deserves further attention.

In conclusion, we have adapted a single excised leaf system for *A. reptans* that effectively uncouples RFO accumulation from low temperature. After physiologically validating that excision does not alter the fundamental carbohydrate metabolism of leaves, we used the system as a tool to demonstrate that frost tolerance in the leaves of *A. reptans* correlates positively with the concentration of accumulated RFOs, suggesting a protective role for these oligosaccharides in the natural frost tolerance of *A. reptans.* Present strategies to understand the role of higher RFO oligomers in frost tolerance are underway in our laboratory. A transformation system for *A. reptans* is being optimized and we envisage that excised leaves from transgenic plants, where GGT has been silenced, will provide key information to understanding the fundamental importance of these higher RFOs in frost tolerance.

## ACKNOWLEDGMENTS

The authors wish to thank Stefan Hörtensteiner for critical reading of the manuscript. This work was supported by a grant from the Swiss National Foundation.

## REFERENCES

Arakawa T., Prestrelski S.J., Kenney W.C. & Carpenter J.F. (2001) Factors affecting short-term and long-term stabilities of proteins. *Advanced Drug Delivery Reviews* **46,** 307–326.

Bachmann M. & Keller F. (1995) Metabolism of the raffinose family oligosaccharides in leaves of *Ajuga reptans* L. Inter- and intracellular compartmentation. *Plant Physiology* **109,** 991–998.

Bachmann M., Matile P. & Keller F. (1994) Metabolism of the raffinose family oligosaccharides in leaves of *Ajuga reptans* L. Cold acclimation, translocation, and sink to source transition: discovery of chain elongation enzyme. *Plant Physiology* **105,** 1335–1345.

Cacela C. & Hincha D.K. (2006) Monosaccharide composition, chain length and linkage type influence the interactions of oligosaccharides with dry phosphatidylcholine membranes. *Biochimica et Biophysica Acta* **1758,** 680–691.

Carpenter J.F. & Crowe J.H. (1989) An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. *Biochemistry* **28,** 3916–3922.

Chinnusamy V., Zhu J. & Zhu J.-K. (2007) Cold stress regulation of gene expression in plants. *Trends in Plant Science* **12,** 444–451.

Cunningham S.M., Nadeau P., Castonguay Y., Laberge S. & Volenec J.J. (2003) Raffinose and stachyose accumulation, galactinol synthase expression, and winter injury of contrasting alfalfa germplasms. *Crop Science* **43,** 562–570.

Gaffney S., Haslam E., Lilley T. & Ward T. (1988) Homotactic and heterotactic interactions in aqueous-solutions containing some saccharides – experimental results and an empirical relationship between saccharide solvation and solute–solute interactions. *Journal of the Chemical Society, Faraday Transactions* **84,** 2545–2552.

Hamman R.A. Jr, Dami, I.E., Walsh T.M. & Stushnoff C. (1996) Seasonal carbohydrate changes and cold hardiness of Chardonnay and Riesling grapevines. *American Journal of Enology and Viticulture* **47,** 31–36.

Haritatos E., Keller F. & Turgeon R. (1996) Raffinose oligosaccharide concentrations measured in individual cell and tissue types in *Cucumis melo* L. leaves: implications for phloem loading. *Planta* **198,** 614–622.

Hatanaka R. & Sugawara Y. (2008) Development of desiccation tolerance and vitrification in cultured cells of *Marchantia polymorpha*. *Cryobiology* **57,** 339.

Hincha D.K., Zuther E. & Heyer A.G. (2003) The preservation of liposomes by raffinose family oligosaccharides during drying is mediated by effects on fusion and lipid phase transitions. *Biochimica et Biophysica Acta* **1612,** 172–177.

Imanishi H.T., Suzuki T., Masuda K. & Harada T. (1998) Accumulation of raffinose and stachyose in shoot apices of *Lonicera caerulea* L. during cold acclimation. *Scientia Horticulturae* **72,** 255–263.

Inan Haab C.I. & Keller F. (2002) Purification and characterization of the raffinose oligosaccharide chain elongation enzyme, galactan : galactan galactosyltransferase (GGT), from *Ajuga reptans* leaves. *Plant Physiology* **114,** 361–371.

Keller F. & Pharr M. (1996) Metabolism of carbohydrates in sinks and sources: galactosyl-sucrose oligosaccharides. In *Photoassimilate Distribution in Plants and Crops: Source-Sink Relationships* (eds E. Zamski & A.A. Schaffer) pp. 157–183. Marcel Dekker, New York, NY, USA.

King R.W. & Zeevaart J.A.D. (1974) Enhancement of phloem exudation from cut petioles by chelating agents. *Plant Physiology* **53,** 96–103.

Klotke J., Kopka J., Gatzke N. & Heyer A.G. (2004) Impact of soluble sugar concentrations on the acquisition of freezing tolerance in accessions of *Arabidopsis thaliana* with contrasting cold adaptation – evidence for a role of raffinose in cold acclimation. *Plant, Cell & Environment* **27,** 1395–1404.

Koster K.L., Lei Y.P., Anderson M., Martin S. & Bryant G. (2000) Effects of vitrified and nonvitrified sugars on phosphatidylcholine fluid-to-gel phase transitions. *Biophysical Journal* **78,** 1932–1946.

Pennycooke J.C., Jones M.L. & Stushnoff C. (2003) Down-regulating $\alpha$-galactosidase enhances freezing tolerance in transgenic petunia. *Plant Physiology* **133,** 901–909.

Peters S., Mundree S.G., Thomson J.A., Farrant J.M. & Keller F. (2007) Protection mechanisms in the resurrection plant *Xerophyta viscosa* (Baker): both sucrose and raffinose family oligosaccharides (RFOs) accumulate in leaves in response to water deficit. *Journal of Experimental Botany* **58,** 1947–1956.

Prestrelski S.J., Tedeschi N., Arakawa T. & Carpenter J.F. (1993) Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. *Biophysical Journal* **65,** 661–671.

Salerno G.L. & Pontis H.G. (1989) Raffinose synthesis in *Chlorella vulgaris* cultures after a cold shock. *Plant Physiology* **89,** 648–651.

Santarius K.A. & Milde H. (1977) Sugar compartmentation in frost-hardy and partially dehardened cabbage leaf cells. *Planta* **136,** 163–166.

Shahba M.A., Qian Y.L., Hughes H.G., Koski A.J. & Christensen D. (2003) Relationships of soluble carbohydrates and freeze tolerance in saltgrass. *Crop Science* **43,** 2148–2153.

Tapernoux-Lüthi E.M., Böhm A. & Keller F. (2004) Cloning, functional expression, and characterization of the raffinose oligosaccharide chain elongation enzyme, galactan : galactan galactosyltransferase, from common bugle leaves. *Plant Physiology* **134,** 1377–1387.

Tapernoux-Lüthi E.M., Schneider T. & Keller F. (2007) The C-terminal sequence from common bugle leaf galactan : galactan galactosyltransferase is a non-sequence-specific vacuolar sorting determinant. *FEBS Letters* **581,** 1811–1818.

Turgeon R. & Gowan E. (1990) Phloem loading in *Coleus blumei* in the absence of carrier-mediated uptake of export sugar from the apoplast. *Plant Physiology* **94,** 1244–1249.

Valluru R., Lammens W., Claupein W. & Van den Ende W. (2008) Freezing tolerance by vesicle-mediated fructan transport. *Trends in Plant Science* **13,** 409–414.

Wiemken V. & Ineichen K. (1993) Effect of temperature and photoperiod on the raffinose content of spruce roots. *Planta* **190,** 387–392.

Zimmerman M. & Ziegler H. (1975) List of sugars and sugar alcohols in sieve-tube exudates. In *Encyclopedia of Plant Physiology* (eds M. Zimmermann & J. Milburn) pp. 480–503. Springer Verlag, Berlin, Germany.

Zuther E., Buchel K., Hundertmark M., Stitt M., Hincha D.K. & Heyer A.G. (2004) The role of raffinose in the cold acclimation response of *Arabidopsis thaliana*. *FEBS Letters* **576,** 169–173.

*Received 21 January 2009; received in revised form 30 March 2009; accepted for publication 1 April 2009*

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AtGolS | *Arabidopsis thaliana* galactinol synthase |
| α-Gal | α-galactosidase enzyme |
| BLAST | basic local alignment search tool |
| CBF/DREB | C-repeat binding/dehydration-responsive element binding |
| cDNA | complementary DNA |
| Col-0 | *Arabidopsis thaliana* ecotype Columbia-0 |
| dNTP | deoxynucleotide triphosphate |
| DP | degree of polymerization |
| DTT | dithiothreitol |
| DW | dry weight |
| EDTA | ethylenediaminetetraacetic acid |
| FW | fresh weight |
| Gal | galactose |
| GGT | galactan:galactan galactosyltransferase |
| Glc | glucose |
| Gol | galactinol |
| GolS | galactinol synthase |
| Hex | hexose (sum of glucose, fructose and galactose) |
| HPLC-PAD | High-performance Liquid Chromatography with Pulsed Amperometric Detector |
| Ino | *myo*-inositol |
| kb | kilobase |
| MS | Murashige and Skoog |
| n.d. | not detected |
| NASC | Nottingham Arabidopsis Stock Centre |
| nkat | nano katal |
| PCR | polymerase-chain-reaction |
| PMSF | phenylmethylsulfonylfluoride |
| PVP | polyvinylpyrrolidone |
| PVPP | polyvinylpolypyrrolidone |
| Raf | raffinose |
| RafS | raffinose synthase |
| RFOs | raffinose family oligosaccharides |
| RNA | ribonucleic acid |
| rRNA | ribosomal RNA |
| RT-PCR | reverse transcriptase-polymerase-chain-reachtion |

| | |
|---|---|
| SDS | sodiumdodecylsulphate |
| SE | standard error |
| Sf9 | insect cell line from *Spodoptera frugiperda* |
| SIP | seed imbibition proteins |
| Sta | stachyose |
| StaS | stachyose synthase |
| Suc | sucrose |
| T-DNA | transferred DNA |
| v/v | volume-to-volume solution |
| Ver | verbascose |

## CURRICULUM VITAE

### Personalien

Name: PETERS

First name: Shaun Wayne

Geboren am: 03.01.1977

Heimatort: Kwazulu-Natal Province, Süd Afrika

### Ausbildung

1994 Westerford High School, Newlands, Cape Town, South Africa.

1995-1998 **Bachelor of Science (BSc.)** Faculty of Science, University of Cape Town, South Africa. Major subjects: Marine biology, Freshwater ecology.

1999 **Post graduate diploma in organization and Management (PDOM),** School of Management Studies, Faculty of Commerce, University of Cape Town, South Africa. Awarded with distinction and class medal.

2000 **BSc. 3rd Major Subject (Microbiology)**, Faculty of Science, University of Cape Town, South Africa

2001 **Bachelor of Science Honours (BSc. Hons.),** Department of Molecular and Cell Biology, University of Cape Town, South Africa.

2002-06/2005 **Masters of Science (MSc.) by dissertation,** Department of Molecular and Cell Biology, University of Cape Town, South Africa.

Dissertation title: *XvGolS*, a galactinol synthase is transcriptionally upregulated under water deficit: The role of raffinosaccharides in abiotic stress tolerance in the resurrection plant *Xerophyta viscosa (*Baker).

2005-2009 **Doctor of Philosophy (Ph.D.)**, Institute of Plant Biology, University of Zürich, Switzerland.

Plant, Cell & Environment

*Plant, Cell and Environment* (2009) **32**, 1099–1107 doi: 10.1111/j.1365-3040.2009.01991.x

# Frost tolerance in excised leaves of the common bugle (*Ajuga reptans* L.) correlates positively with the concentrations of raffinose family oligosaccharides (RFOs)

SHAUN PETERS & FELIX KELLER

*University of Zürich, Institute of Plant Biology, Molecular Plant Physiology, Zollikerstrasse 107, CH-8008 Zürich, Switzerland*

## ABSTRACT

**Mass increases in raffinose family oligosaccharides (RFOs, α1,6-galactosyl extensions of sucrose) are well documented in the generative tissues of many plants upon cold acclimation, and they (i.e. mainly the two shortest RFO members, raffinose and stachyose) have been suggested as frost stress protectants. Our focus here was on the longer RFO members as they commonly occur in the frost-hardy evergreen labiate *Ajuga reptans* in its natural habitat, and accumulate to their highest concentrations in winter when the plant is faced with sub-zero temperatures. We examined the effects of RFO concentration and chain length on frost tolerance using excised leaves which accumulate long-chain RFOs under both cold and warm conditions, thereby uncoupling the acclimation temperature from RFO production. We demonstrated that frost tolerance in excised *A. reptans* leaves correlates positively with long-chain RFO accumulation under both acclimation temperatures. After 24 d post-excision in the warm, the leaves had increased their RFO concentrations (mainly long-chain RFOs) 22-fold to 78 mg $g^{-1}$ fresh weight, and decreased their $EL_{50}$ values (temperature at which 50% leakage occurred) from –10.5 to –24.5 °C, suggesting a protective role for these oligosaccharides in the natural frost tolerance of *A. reptans*.**

*Key-words*: cold acclimation; electrolyte leakage; freezing; water soluble carbohydrates.

## INTRODUCTION

Raffinose family oligosaccharides (RFOs; Suc-$[Gal]_n$, $13 < n \geq 1$) are α1,6-galactosyl extensions of sucrose (Suc) that occur frequently in higher plants. The RFO biosynthetic pathway is initiated with the synthesis of the galactosyl donor galactinol (Gol; 1-O-α-D-galactopyranosyl-L-*myo*-inositol), catalysed by the enzyme galactinol synthase (GolS, EC 2.4.1.123) using UDP-Gal and *myo*-inositol as substrates. Subsequently, the α-galactosyltransferase raffinose (Raf) synthase (RafS, EC 2.4.1.82) transfers a galactosyl moiety from Gol to the $C_6$ position of the glucose (Glc) moiety in Suc, forming an α1,6-galactosidic linkage to yield the trisaccharide Raf. Similarly, stachyose (Sta) synthase (StaS, EC 2.4.1.67) transfers a galactosyl moiety from Gol to the $C_6$ position of the Gal moiety in Raf to yield the tetrasaccharide Sta. In *Ajuga reptans*, a Gol-independent biosynthetic pathway has been reported for higher RFO oligomer biosynthesis (Bachmann & Keller 1995; Haab & Keller 2002; Tapernoux-Lüthi, Böhm & Keller 2004). The enzyme galactan : galactan galactosyltransferase (GGT) utilizes RFOs as galactosyl donors and acceptors during chain elongation, facilitating the synthesis of higher RFO oligomers (up to Suc-$[Gal]_{13}$) in *A. reptans*.

Together with Suc, RFOs have been well characterized as having functional roles in carbon translocation and storage in a number of plant families (Zimmerman & Ziegler 1975; Keller & Pharr 1996). Mass increases in RFOs have also been correlated with the onset of low temperature in a number of plants including alfalfa (Cunningham *et al.* 2003), *Arabidopsis* (Klotke *et al.* 2004), cabbage (Santarius & Milde 1977), salt grasses (Shahba *et al.* 2003), spruce (Wiemken & Ineichen 1993) as well as the photoautotrophic alga *Chlorella vulgaris* (Salerno & Pontis 1989). A recent report has also indicated that, in petunia plants, where an α-galactosidase (α-Gal) had been down-regulated (the first committed step in RFO hydrolysis, catalyzing the cleavage of the terminal α-linked galactose), Raf hyperaccumulation was correlated positively with an increase in frost tolerance (Pennycooke, Jones & Stushnoff 2003). Collectively, such observations firmly place RFOs as having a functional role in low temperature stress. All RFOs share a fundamental characteristic with compatible solutes; as nonreducing sugars, they can accumulate to high intracellular concentrations without affecting metabolic processes. Presumably all RFOs, including higher oligomers, may thus exert protective effects that expand their functional significance (as phloem and storage carbohydrates) to include stress protection.

We have had a long standing research interest in the RFO metabolism of the frost tolerant, evergreen labiate *A. reptans*, and have thus far reported that in this plant: (1) Sta is the main carbon translocate; (2) higher RFO oligomers are the main carbon store (Bachmann, Matile & Keller

*Correspondence: F. Keller. Fax: +41 44 634 8204; e-mail: fkel@botinst.uzh.ch*

1994); and (3) higher RFO oligomers are synthesized by GGT which is targeted to the vacuole via a novel sorting determinant (Bachmann *et al.* 1994; Haab & Keller 2002; Tapernoux-Lüthi *et al.* 2004; Tapernoux-Lüthi, Schneider & Keller 2007). We have also long speculated that the frost tolerant nature of this plant is associated with the high concentrations of RFOs it contains during autumn and winter [up to 200 mg $g^{-1}$ fresh weight (FW) in leaves; Bachmann *et al.* 1994], but have thus far never undertaken to quantify frost resistance with respect to varying RFO concentrations and chain lengths.

The importance of the lowest RFO oligomer, Raf, in the low temperature acclimation and frost tolerance of *Arabidopsis* has recently been challenged (Zuther *et al.* 2004). We were thus interested in analysing the effects of RFO concentration and chain length in the frost tolerance of *A. reptans*. In whole, soil-grown plants, we are only able to induce the accumulation of RFOs through cold acclimation (8/3 °C, 16 h/8 h). Typically, RFOs begin accumulating after 4–6 weeks of acclimation. Given that a number of other low temperature-induced protective pathways come into play during long-term cold acclimation (for review see Chinnusamy, Zhu & Zhu 2007), dissecting a functional role for RFOs in the frost tolerance of *A. reptans* is challenging.

Our aim was, therefore, to employ a system that would effectively uncouple RFO accumulation from low temperature acclimation, thereby allowing us to analyse frost tolerance as a function of RFO concentration. To this end, it has been previously demonstrated that excised leaves of *A. reptans*, placed in water-filled test tubes, rapidly accumulate long-chain RFOs and increase GGT activity in the warm (Haab & Keller 2002; Tapernoux-Lüthi *et al.* 2007). Here, we report on the refinement of the excised leaf system, describing its physiological validation and use as a tool to analyze concentration-dependent effects of RFOs on frost tolerance in *A. reptans*. We demonstrate that: (1) excision does not affect the fundamental carbohydrate physiology of a leaf; and (2) the frost tolerance of excised leaves is improved as RFO concentration increases, suggesting a protective role for RFOs in the natural frost tolerance of *A. reptans*.

## MATERIALS AND METHODS

### Plant material

*Ajuga reptans* plants were grown in a controlled environment chamber (12 h light, 30 $\mu$mol photons $m^{-2}$ $s^{-1}$, 22 °C, 12 h dark, 60% RH) in 6.5 L of Luwasa® hydroculture medium (0.3% v/v, Interhydro AG, Allmendingen, Switzerland) which was continuously aerated using a home aquarium air pump. Growth medium was replaced weekly. Plants were considered experimentation-ready once the root systems had reached approximately 20 cm in length and leaf rosettes were abundant. *Nicotiana benthamiana* plants were propagated from seeds under greenhouse conditions. After 7 d, plants were transplanted: one to a pot and transferred into the growth chamber described above. Plants were considered experimentation-ready after a further 7 d of growth in the chamber.

### Leaf sets

Mature source leaves of similar size were excised from rosettes with a scalpel and the ends of the petioles subsequently re-cut under water. Petioles were immersed in a test tube, filled with water and capped with a perforated black plastic cap that allowed the leaf petiole through. Leaf sets (six leaves per set) were then placed in a test tube rack covered with black PVC sheeting, exposing only the leaf to light and maintained in the controlled environment chamber described above. One leaf set was subsequently sampled every 6 d over a period of 30 d and processed for carbohydrate and enzyme extractions, as well as frost tolerance experiments. *N. benthamiana* leaves were treated in the same manner, except that leaf sets were sampled at 6, 9 and 12 d.

### Water soluble carbohydrate (WSC) extraction

WSCs were extracted using an ethanol series, as previously described (Peters *et al.* 2007) with modifications. Two leaf discs (6 mm ∅) were punched out of each leaf in a set, weighed, flash-frozen in liquid $N_2$ and macerated using a plastic pestle in a 1.5 mL Eppendorf tube. WSCs were extracted twice (per step) in a three-step sequential process, using 100 $\mu$L 10 mg $g^{-1}$ FW of 80% EtOH, 50% EtOH and $dH_2O$. Extractions were conducted at 85 °C for 10 min and the tubes centrifuged at 12 000 *g* (5 min, 4 °C). Supernatants were removed to a separate Eppendorf tube before the next extraction in the sequence. The supernatants of all extractions were pooled and concentrated in a vacuum concentrator centrifuge. Extracts were then re-suspended in 100 $\mu$L of $dH_2O$. Aliquots (50 $\mu$L) were de-ionized and de-phenolized as described below, and analysed by high-performance liquid chromatography with pulsed amperometric detection (HPLC-PAD).

### Enzyme extractions and activity assays

#### *Plant crude extracts*

Freshly harvested leaf material (200 mg) was ground in 500 $\mu$L of chilled extraction buffer A for alkaline enzyme extracts [50 mM Hepes/KOH pH 7.5, 5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 20 mM DTT, 0.1% (v/v) Triton X-100, 1 mM benzamidine, 1 mM PMSF, 50 mM Na ascorbate, 2% (w/v) PVP] or extraction buffer B for acidic extracts [50 mM trisodium citrate, 20 mM DTT, 5 mM $MgCl_2$, 2% PEG 6000 (w/v), 2% PVP K30 (w/v) pH 5.0]. Samples were transferred into 2 mL Eppendorf tubes and centrifuged at 12 000 *g* (10 min, 4 °C). A 150 $\mu$L aliquot of supernatant was desalted by gel filtration at 1400 *g* (2 min, 4 °C) through 5 mL Sephadex G-25 columns (fine, final bed volume of 3 mL). Columns were pre-equilibrated with an alkaline assay buffer (50 mM Hepes/

KOH pH 7.5, 2 mM $MnCl_2$, 10 mM DTT) or an acidic assay buffer (McIlvaine Buffer pH 5.0). Pre-equilibration was performed twice by centrifugation at 1400 *g* (2 min, 4 °C), each with 2 mL of assay buffer. Aliquots (20 $\mu$L) of desalted extract were assayed for respective enzyme activities in a final volume of 40 $\mu$L assay buffer containing 100 mM Suc and 10 mM Gol for RafS, 50 mM Raf and 5 mM Gol for StaS, 100 mM Raf for GGT, and both acidic and alkaline $\alpha$-Gals. All assays were conducted for 1 h at 30 °C and stopped by flash-freezing the tubes in liquid $N_2$, and subsequently boiling for 5 min. Samples were de-ionized, de-phenolized and analysed by HPLC-PAD.

### *Desalting of extracts*

Desalting of carbohydrate and enzyme assay samples to remove phenolics and ions was conducted by centrifuge-rinsing of the samples through pre-rinsed 1 mL Mobicol spin columns (MoBiTec, Göttingen, Germany), as previously described (Peters *et al.* 2007) with minor modifications. Aliquots of ethanol extracts (75 $\mu$L) were desalted and centrifuge-rinsed twice, each with 175 $\mu$L of $dH_2O$. Desalted samples were then concentrated in a vacuum concentrator centrifuge and re-suspended in 100 $\mu$L of $dH_2O$ prior to HPLC-PAD. All 40 $\mu$L of enzyme assay reactions were desalted and centrifuge-rinsed once with 100 $\mu$L of $dH_2O$.

### *HPLC-PAD analysis*

Desalted carbohydrate extracts and enzyme assay reactions were analysed and quantified by HPLC-PAD as described in Peters *et al.* (2007). Briefly, two chromatographic systems using either a $Ca^{2+}/Na^{+}$-moderated ion partitioning carbohydrate column (Benson BC100, BC200 columns, 7.8 × 300 mm; Benson Polymeric, Reno, Nevada, USA), or an anion exchange CarboPac PA1 column (4 × 250 mm; Dionex, Sunnyvale, CA, USA) were used to separate carbohydrates. Quantification was done using the Chromeleon v6.4 software package against a series of 5 nmol of standard sugars, the concentration of which corresponded to the linear response range of both chromatographic systems. In the absence of commercially available higher RFO oligomer standards, long-chain RFOs (DP, degree of polimerization, > 5) were quantified against a verbascose (Ver; DP5) standard and are presented as Ver equivalents.

### *Pulse-chase experiments*

Six excised leaves were maintained in the controlled environment chamber described above for 6 d, then placed in a glass thin layer chromatography chamber. Two 1.5 mL Eppendorf tubes were scotch taped to the sidewalls. To introduce $^{14}CO_2$ into the system, $NaH^{14}CO_3$ (Hartmann Analytic, Braunschweig, Germany) was added to the Eppendorf tubes (0.925 MBq $g^{-1}$ FW, specific activity 2183 MBq $mmol^{-1}$). Lactic acid was added to each of the tubes and the chromatography tank was sealed with a glass lid lined with silicone grease. Leaves were subjected to strong illumination (460 $\mu$mol photons $m^{-2}$ $s^{-1}$) for 15 min and chased with $^{12}CO_2$ for 2 h, on the benchtop, without additional illumination. Leaves were then processed either for carbohydrate extractions described above or phloem exudation.

### *Phloem exudations*

Phloem exudations were based on the method previously described (King & Zeevaart 1974; Bachmann *et al.* 1994), with modifications. After pulsing leaves with $^{14}CO_2$, petiole ends were re-cut under exudation buffer (5 mM EDTA, 5 mM $KHPO_4$ pH 7.0) and placed in an Eppendorf tube filled with exudation buffer. Exudations were allowed to proceed for 2–4 h in a sealed, humidified chromatography chamber. Subsequently, the samples were analysed for $^{14}C$-carbohydrates by separation on a BC100 column coupled to a FLO-ONE radio chromatography detector (Model A-525X, Packard, Zürich, Switzerland).

### *Frost tolerance experiments*

Leaf sets were harvested at various post-excision times (0, 6, 12, 18 and 24 d) and subjected to freezing temperatures of –5, –10, –15 and –20 °C in a programmable freezing chamber (RUMED, Blanc-Labo S.A., Lonay, Switzerland). Prior to being placed in the chamber, leaves were transferred into plastic bags containing a few ice chips and the end of the bags tied off loosely with string. For each freezing temperature, the chambers were programmed to decrease (from –1 °C) by –1 °C $h^{-1}$ and hold at the designated temperature for 3 h before increasing by +1 °C $h^{-1}$ until the hold temperature of –1 °C was reached. Subsequently, the plastic bags were placed on ice to thaw. Two leaf discs (6 mm ∅) were punched out of each leaf. Discs were placed immediately into the wells of a six-well microtiter plate and incubated at room temperature for 1 h with agitation. Each well contained 1.5 mL of de-ionized water. The conductivity was measured for each well using a conductivity meter (Model 712, Metrohm, Zofingen, Switzerland) and this represented the initial leakage ($L_i$). Leaf discs were then flash-frozen in liquid $N_2$, quickly re-immersed in the same well they had originally been in and incubated for 1 h as described above. Conductivity was measured for each well and this represented the total leakage ($L_t$). Electrolyte leakage was expressed as a relative percentage of total leakage [($L_i$ $L_t^{-1}$) ∗ 100]. The temperature at which 50% relative leakage occurred from these data was defined as the $EL_{50}$. For each series of inductions, $EL_{50}$ values were obtained by plotting non-linear (sigmoidal) regression curves through scatter plots of the relative leakage data obtained for each freezing temperature.

## RESULTS

### The leaves of *A. reptans* plants exhibit two distinct RFO states with differing frost tolerance

HPLC-PAD analysis of total leaf WSCs indicated that warm-grown plants (22 °C) accumulated proportionally

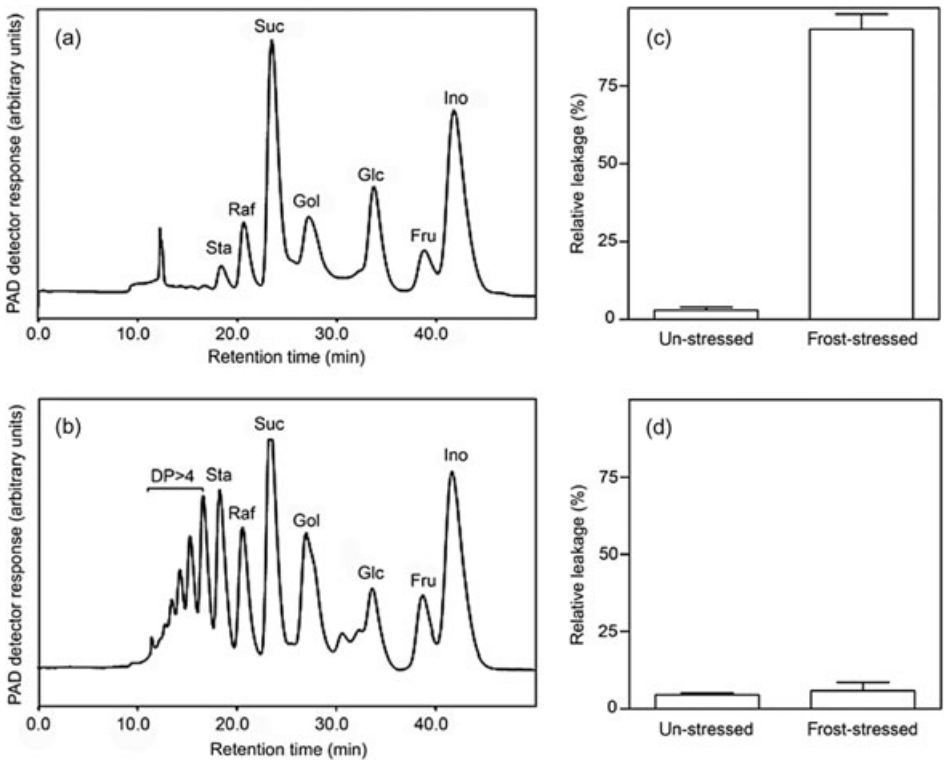


**Figure 1.** Water soluble carbohydrates (WSCs) and frost tolerance of leaves from warm-grown (22 °C; a, c) and cold-grown (8/3 °C, day/night; b, d) *Ajuga reptans* plants. Graphs a and b represent the WSC profiles, while graphs c and d represent the relative electrolyte leakage from leaves after exposure to a freezing stress of –20 °C. Data points represent the mean ± SE of six replicates. Samples were analysed by high-performance liquid chromatography using the BC200 column. Fru, fructose; Glc, glucose; Gol, galactinol; Ino, *myo*-inositol. PAD, pulsed amperometric detection; Raf, raffinose; Sta, stachyose; Suc, sucrose.

lower amounts of total RFOs, with Sta (DP4) being the highest RFO oligomer detectable (Fig. 1a). In cold-grown plants (8 °C/3 °C, day/night), RFOs were proportionally higher and were detectable as higher oligomers up to about DP9 (Fig. 1b). When leaves from both of these plants were exposed to a freezing stress of –20 °C, relative electrolyte leakage differed greatly. In the leaves of warm-grown plants, it was >90%, although it was 6% in the leaves of cold-grown plants, comparable to the relative leakage of un-stressed, warm-grown control leaves (Fig. 1c & d, respectively).

## Excised leaves produce mainly $^{14}$C-Suc as a photosynthate and export mainly $^{14}$C-Sta as a phloem translocate in $^{14}CO_2$ photosynthetic pulse-chase experiments

Photosynthetic pulse-chase experiments using $^{14}CO_2$ were conducted with excised leaves at 6, 12 and 24 d after excision (DAE). Radio-HPLC analyses of the leaf WSCs indicated that the major proportion of photosynthetically fixed carbon at all time points consistently occurred as $^{14}$C-Suc, along with $^{14}$C-Raf, $^{14}$C-Sta, $^{14}$C-Gol, $^{14}$C-Glc and $^{14}$C-Fru (fructose) (Fig. 2a, representative, 12 DAE). Radio-HPLC analyses of the EDTA phloem exudates from these leaves indicated that the predominant $^{14}$C-WSC in the phloem sap was $^{14}$C-Sta (Fig. 2b, representative, 12 DAE).

## Warm-incubated (22 °C) excised leaves accumulate RFOs independent of cold acclimation

The changes in WSC concentrations were analysed in warm-incubated excised leaves at 6 d intervals over a period of 30 d. The total RFO concentration increased nearly 22-fold to 82 mg g$^{-1}$ FW (Fig. 3). Higher RFO oligomers (DP > 4) accounted for the largest portion, showing a 74-fold increase from 0.6 to 57 mg g$^{-1}$ FW after 30 d. This correlated positively with an eightfold increase in GGT activity from 5.6 to 46.4 nkat g$^{-1}$ FW. Sta increased 7.7-fold from 1.8 to 13.7 mg g$^{-1}$ FW. Raf increased sevenfold over the same period from 1.1 to 8 mg g$^{-1}$ FW. A clear positive correlation between Raf and RafS activity was not evident.

Suc concentration was nearly constant, showing a relatively minor 1.4-fold increase from 3.2 to 4.5 mg g$^{-1}$ FW (Fig. 3). Activities for RFO hydrolytic enzymes indicated that alkaline $\alpha$-Gal activity did not change over the 30 d period. However a pronounced increase in acidic $\alpha$-Gal activity occurred between 18 and 30 DAE (Fig. 3). Together with control leaves, we chose leaves at 12 and 24 DAE to represent low, intermediate and high RFO states,

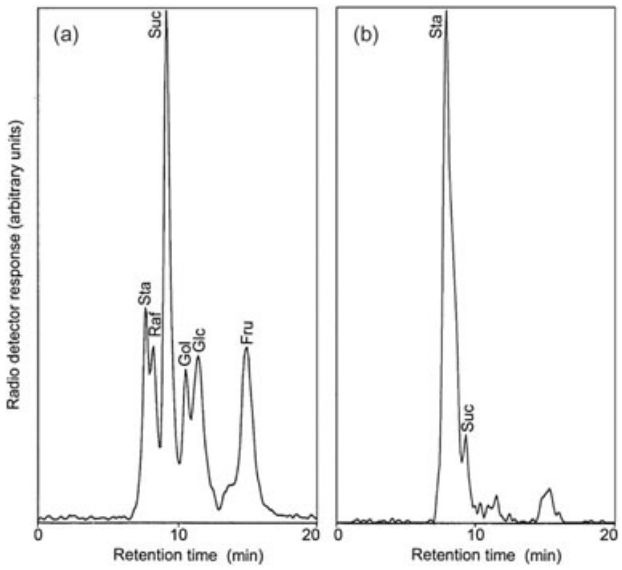


**Figure 2.** Representative radio high-performance liquid chromatography of $^{14}$C-WSCs $^{14}CO_2$ photosynthetic pulse-chase experiments conducted on warm-incubated (22 °C) *Ajuga reptans* excised leaves (12 DAE), and analysed for carbohydrates (a) and phloem exudation (b). Samples were analysed using the BC100 column. DAE, days after excision; Fru, fructose; Glc, glucose; Gol, galactinol; Raf, raffinose; Sta, stachyose; Suc, sucrose; WSC, water soluble carbohydrates.

respectively, to be subjected to frost tolerance experiments as described.

### Electrolyte leakage and $EL_{50}$ are lower in leaves with high RFO concentration

Excised leaves from 12 and 24 DAE were exposed to freezing temperatures of –5 to –20 °C. Leaves at 24 DAE displayed the lowest relative electrolyte leakage over all temperatures tested (Fig. 4). Similarly, leaves at 12 DAE showed an intermediate relative leakage between that of the control and 24 DAE. Temperatures of –15 and –20 °C resulted in similar average relative leakage of 75% for controls, 60% for 12 DAE and 45% for 24 DAE, suggesting that –15 °C is the upper limit of tolerance for leaves at these RFO states.

Excised leaves with a low RFO concentration (controls) had an $EL_{50}$ of about –10.5 °C (Fig. 5). As RFO concentrations in excised leaves increased, the $EL_{50}$ temperature decreased further with leaves showing an $EL_{50}$ of –16.0 °C at 12 DAE and –24.5 °C at 24 DAE (Fig. 5). The strongest correlation between $EL_{50}$ and RFO concentration was evident for higher RFO oligomers (>Sta). Excised leaves of *N. benthamiana* had no detectable RFOs over 12 DAE when incubated under both cold and warm conditions. The frost tolerance of leaves incubated in the warm did not improve over this period, with leaves 12 DAE showing a slightly higher (75%) relative electrolyte leakage than leaves 6 DAE, when challenged by freezing at –5 °C (50%, Fig. 4b). Excised *N. benthamiana* leaves incubated in the cold showed a marked improvement in frost tolerance 6 DAE, compared with control leaves with relative electrolyte leakage of about 10% at –5 °C but this tolerance did not improve over 12 DAE.

## DISCUSSION

### *A. reptans* plants exist in two distinct physiological states that differ in frost tolerance

*Ajuga reptans* exists naturally in two distinct physiological states relative to RFO pools. It has been reported that during spring and summer in Zürich (April to August), RFO concentrations in this plant are relatively low, attaining concentrations of 74 and 42 mg $g^{-1}$ FW in aerial parts and roots, respectively (Bachmann *et al.* 1994). In the transition from late summer to autumn (late August), dramatic mass increases in RFOs occur, attaining maximum concentrations of 200 and 110 mg $g^{-1}$ FW in aerial parts and roots, respectively. Presumably, this represents primarily the storage function of the total RFO pool, as concentration decreases occur in aerial parts and roots throughout late winter to summer (February to June), suggesting a remobilization of the storage RFO pool towards growth in this perennial plant.

Our work on the RFO physiology of *A. reptans* has long pointed towards a second function of these oligosaccharides, i.e. in frost tolerance. The highest RFO concentrations coincide with the winter season, when plants are exposed to the most extreme of low temperatures in Zürich (average day temperature of between 0 and –5 °C, http://www.meteoschweiz.ch). Given that RFO mass increases up to at least Sta have been observed to occur in plants exposed to low temperature (Cunningham *et al.* 2003; Shahba *et al.* 2003; Klotke *et al.* 2004), we quantified the frost tolerance in leaves of cold-grown (8/3 °C, day/night) and warm-grown (22 °C) *A. reptans* plants.

The leaves of warm-grown plants accumulated proportionally lower amounts of RFOs, with Sta being the highest oligomer observed. The leaves of cold-grown plants, however, accumulated proportionally higher amounts of RFOs, with RFOs accumulating to higher oligomers (up to Suc-$[Gal]_7$, Fig. 1). Importantly, after a –20 °C freezing stress, the leaves of cold-grown plants displayed a low relative electrolyte leakage that was comparable to un-stressed control leaves. Leaves of warm-grown plants showed high relative leakage values of >90%, demonstrating that leaves from cold-grown plants are more frost tolerant. To further dissect a role for RFOs in this tolerance, we refined a previously described excised leaf system that would effectively uncouple RFO accumulation from cold acclimation (Haab & Keller 2002).

### Long-term cold acclimation of excised leaves results in additive frost tolerance

Traditionally, we have induced RFO accumulation by exposing whole plants to a low-temperature growth regime

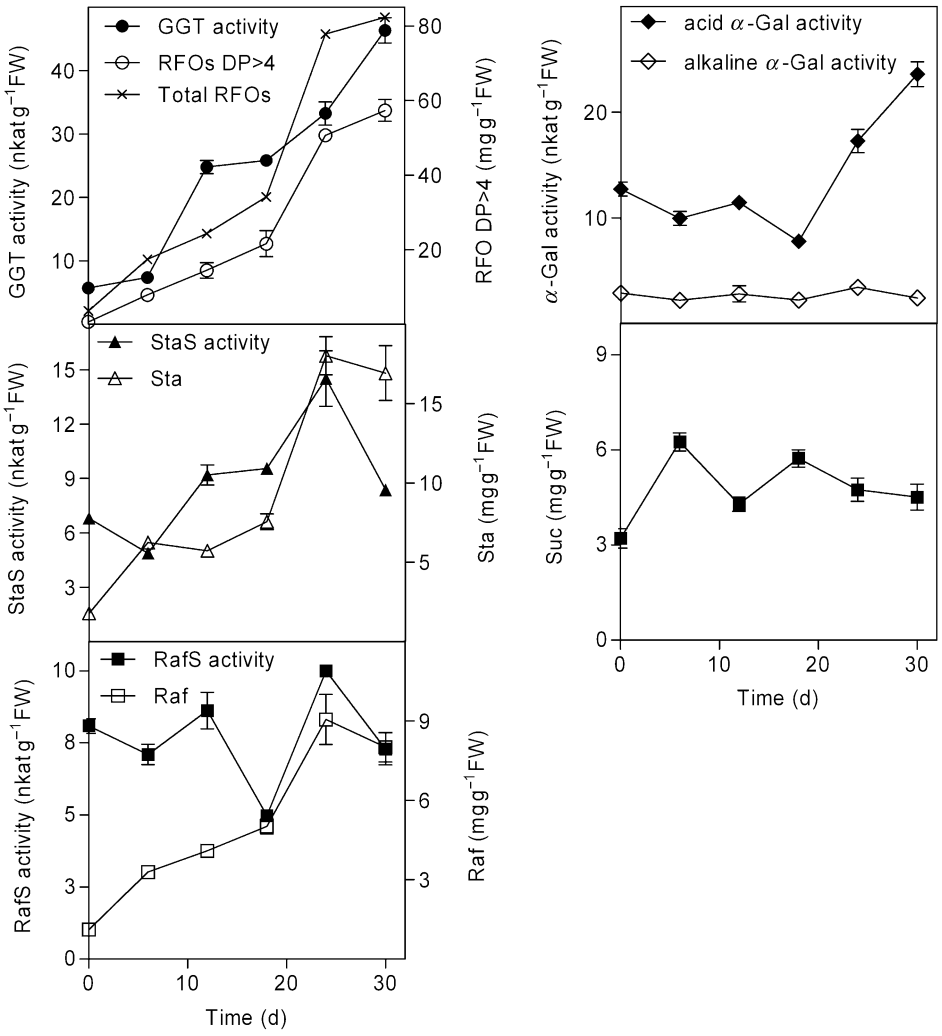


**Figure 3.** WSC and enzyme activity changes in warm-incubated (22 °C) *Ajuga reptans* excised leaves over a 30 d period. Data points represent the mean ± SE of six replicates. Enzyme assay samples were analysed by high-performance liquid chromatography using the BC100 column, while water soluble carbohydrates samples were analysed using both the PA1 and the BC100 columns. α-Gal, α-galactosidase; GGT, galactan : galactan galactosyltransferase; Raf, raffinose; RafS, raffinose synthase; Sta, stachyose; StaS, stachyose synthase; Suc, sucrose.

(8–10/3 °C, day/night) to mimic the transition from late summer to autumn, when *A. reptans* naturally accumulates large amounts of RFOs (Bachmann *et al.* 1994). However, any attempt to analyse a functional role for RFOs in frost tolerance under this regime is met with the problem of the plethora of regulons induced during low temperature (Chinnusamy *et al.* 2007). Cold-incubated excised *A. reptans* leaves accumulate slightly less soluble sugars, in totality, than warm-incubated ones (Haab & Keller 2002). However at 18 DAE, cold-incubated excised leaves are more resistant to –20 °C than warm-incubated ones (Fig. 6). A traditional interpretation of such data would immediately discount a role for soluble sugars in frost tolerance. We, however, maintain that under conditions of long-term cold acclimation, additive protective effects can mask the functional role/s of single components such as RFOs. To use an excised leaf system to examine the role of RFOs in the frost tolerance of *A. reptans*, we first examined the effects of excision on carbohydrate metabolism in leaves and compared these data to that of whole plants.

## Excised leaves produce $^{14}$C-Suc and export $^{14}$C-Sta

The physiological validity of using an excised leaf system to uncouple RFO accumulation from cold acclimation in *A. reptans* was tested by inducing RFO accumulation over a period of 30 d under ambient growth conditions (22 °C). Using classical photosynthetic $^{14}CO_2$ pulse-chase experiments, we demonstrated that at 6, 12 and 24 DAE, single excised leaves operate within normal photosynthetic parameters, producing $^{14}$C-Suc as the major photosynthate, aside from about 10% each of $^{14}$C-Gol, $^{14}$C-Raf and $^{14}$C-Sta (Fig. 2a, representative, 12 DAE). This $^{14}$C-photosynthate

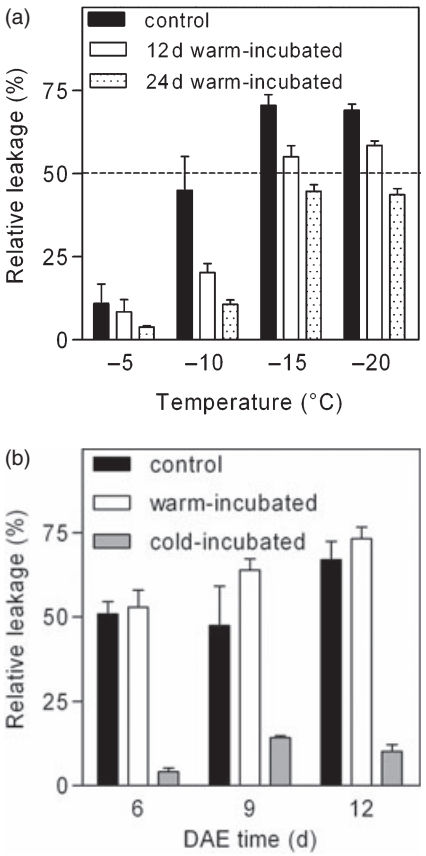


**Figure 4.** Relative electrolyte leakage of (a) warm-incubated (22 °C) *Ajuga reptans* excised leaves exposed to freezing temperatures of between –5 and –20 °C, and (b) warm-incubated (22 °C) and cold-incubated (8/3 °C, day/night) *Nicotiana benthamiana* excised leaves exposed to –5 °C. Control leaves were excised directly from warm-grown plants and subjected to freezing. Data points represent the mean ± SE of six replicates. DAE, days after excision.

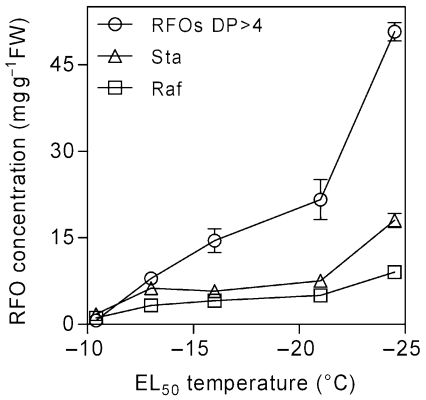


**Figure 5.** $EL_{50}$ values plotted as a function of raffinose family oligosaccharides (RFO) concentration increases in warm-incubated (22 °C) *Ajuga reptans* excised leaves over a 30 d period. Data points represent the mean ± SE of six replicates at 6 d intervals. DP, degree of polymerization; FW, fresh weight; Raf, raffinose; Sta, stachyose.

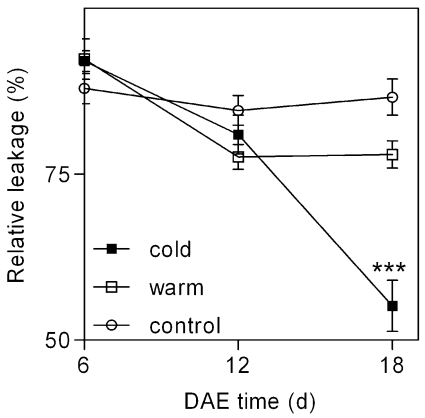


**Figure 6.** Relative electrolyte leakage of warm-incubated (22 °C) and cold-incubated (8/3 °C, day/night) *Ajuga reptans* excised leaves exposed to a freezing stress of –20 °C. Control leaves were excised directly from in warm-grown (22 °C) plants and subjected to freezing at –20 °C. Data points represent the mean ± SE of six replicates. Statistical significance of a two-tailed *t*-test is represented by stars ($P < 0.05$). DAE, days after excision.

pattern is quite similar to that obtained earlier using leaf discs and strips freshly isolated from *A. reptans* leaves (Bachmann & Keller 1995). Furthermore, analysis of phloem exudates after pulsing excised leaves with $^{14}CO_2$ in the light indicated that the major WSC in the phloem is $^{14}$C-Sta (Fig. 2b, representative, 12 DAE), as we have previously reported using *A. reptans* whole plants (Bachmann *et al.* 1994). By removing a leaf from the plant, the major physiological change that we propose to occur is the channelling of newly fixed carbon to the mesophyll vacuolar storage pool (Bachmann & Keller 1995), without any measurable effect on (1) photosynthesis and (2) the ability of the leaf to re-establish phloem transport through an incubation of the petiole excision zone in 5 mM EDTA. Obviously, the possibility of additional effects of leaf excision on frost tolerance cannot be totally excluded but might be minimal.

## Excised leaves accumulate RFOs under warm growth conditions

As reported previously, RFOs increase in warm-incubated excised leaves (Haab & Keller 2002). We have also measured GGT, RafS and StaS activities, showing that GGT activity increases over 30 d correlate with the large increases in higher RFO oligomer concentrations (Fig. 3). Activity measurements for $\alpha$-Gals, catalysing the first committed RFO degradation step, indicated that while alkaline $\alpha$-Gal activity remained unchanged and low over a 30 d period, a very pronounced increase in acid $\alpha$-Gal activity was observed after 18 DAE (Fig. 3). This could be correlated with the emergence of root buds at the excision zone of petioles, observed after 12–14 DAE. Assuming that this temporal window represents the point at which an excised leaf begins to reactivate its source activity, we postulate that

this developmental switch triggers re-mobilization of the RFOs in the mesophyll vacuoles and phloem export of carbon to fuel new root growth.

We have previously shown the phloem loading mechanism in *A. reptans* to be in accordance with the polymer trap model of symplastic loading (Turgeon & Gowan 1990; Bachmann & Keller 1995). This would imply that higher RFO oligomers in the vacuoles are re-mobilized by hydrolysis to Suc, which then diffuses into intermediary cells to be immediately re-synthesized to Raf and Sta, and exported via the phloem from the sieve elements. Unsurprisingly, Suc concentration remained relatively constant compared with RFOs, highlighting the fundamental physiology of *A. reptans*, where post-photosynthetic carbohydrate metabolism is primed to favour RFO biosynthesis.

## Frost tolerance in excised leaves correlates positively with RFO concentration

Most importantly, we have obtained a clear positive correlation between RFO concentrations and the frost tolerance of excised leaves in the absence of cold acclimation. All control leaves had the lowest total RFO concentration (3.6 mg $g^{-1}$ FW) and were tolerant to –5 °C, as evidenced by low relative electrolyte leakage (Fig. 4).

Total leaf RFO concentration increased nearly 22-fold in excised leaves in the warm, 24 DAE, and leaves at 12 and 24 DAE were much more tolerant to freezing temperatures of up to –20 °C than control leaves. The total RFO increase correlated positively with a shift in the $EL_{50}$ value from about –10.5 °C (control) to –24.5 °C (Fig. 5), showing for the first time quantitatively that, in *A. reptans* leaves, RFOs may improve frost tolerance irrespective of cold acclimation. The best correlation to the shift in $EL_{50}$ values was evident between concentration increases in higher RFO oligomers, which accounted for 65% of the total RFO increase. As a control, we also examined the effects of leaf excision on frost tolerance, using excised leaves of *N. benthamiana*. Our HPLC-PAD analysis indicated that no RFOs occurred in either warm- or cold-incubated excised leaves up to 12 DAE, in accordance with previous reports for tobacco (Haritatos, Keller & Turgeon 1996). Importantly, excised *N. benthamiana* leaves incubated in the warm did not show any improvement in frost tolerance (Fig. 4b), despite a 3.5-fold Suc increase to about 418 mg $g^{-1}$ FW 12 DAE. A similar Suc increase (3.4-fold) was evident in cold-incubated excised *N. benthamiana* leaves to about 384 mg $g^{-1}$ FW. However, frost tolerance was markedly improved in cold-incubated leaves 6 DAE, suggesting that other factors apart from Suc may be important in cold acclimation-induced frost protection of *N. benthamiana* excised leaves.

One of the *in vitro* experimentally demonstrated functions of the lowest RFO oligomer, Raf, is its ability to hydrogen bond to biomolecules more effectively than either Suc or trehalose, considered to be the most abundant stress protective sugars in nature (Gaffney *et al.* 1988). This direct hydrogen bonding between sugars and biomolecules has been demonstrated to be imperative, even in low concentrations, in the stabilization of proteins, membranes and whole cells under conditions of freezing and dehydration (Carpenter & Crowe 1989; Prestrelski *et al.* 1993; Koster *et al.* 2000; Arakawa *et al.* 2001; Hincha, Zuther & Heyer 2003; Hatanaka & Sugawara 2008).

The protective efficacy of higher RFO oligomers in frost tolerance cannot be precluded on the basis of the compatible solute nature of these non-reducing sugars. Indeed, there is *in planta* evidence that together with Raf, Sta may play an important role in cold hardiness of *Loniceria caerula* L. shoot apices (Imanishi *et al.* 1998), alfalfa roots (Cunningham *et al.* 2003), as well as Chardonnay and Riesling grapevines (Hamman *et al.* 1996). From *in vitro* studies, it has been reported that commercially available RFO oligomers (up to Ver) offer increasing protection to artificial liposomes, during drying, with increasing chain length (Hincha *et al.* 2003). Similar *in vitro* observations were made for inulin-type fructan oligomers ($\beta$2,1-fructosyl extensions of Suc, Suc-$[Fru]_n$) where lyoprotection was positively correlated with increasing chain length (Cacela & Hincha 2006). Our data has provided convincing *in planta* evidence that frost tolerance in *A. reptans* leaves may depend on RFO concentration, as the excised leaf system uncouples RFO accumulation from cold acclimation. Furthermore, the best positive correlation to increases in $EL_{50}$ were evident for higher RFO oligomers (>Ver), suggesting that they may be important contributors to frost tolerance in this plant. Because these higher RFO oligomers are synthesized and stored in the large central vacuole of mesophyll cells (Bachmann & Keller 1995), the question arises if they may exert their protective role also outside the vacuole, e.g. in the plasma membrane. To do so, they would need to reach the plasma membrane, but no such mechanism has been described for RFOs nor has their putative apoplastic location been determined. The recently proposed model for vesicle-mediated transport of fructans from the vacuole to the apoplast to assist in stabilizing the plasma membrane (Valluru *et al.* 2008) might also be applicable to RFOs and deserves further attention.

In conclusion, we have adapted a single excised leaf system for *A. reptans* that effectively uncouples RFO accumulation from low temperature. After physiologically validating that excision does not alter the fundamental carbohydrate metabolism of leaves, we used the system as a tool to demonstrate that frost tolerance in the leaves of *A. reptans* correlates positively with the concentration of accumulated RFOs, suggesting a protective role for these oligosaccharides in the natural frost tolerance of *A. reptans.* Present strategies to understand the role of higher RFO oligomers in frost tolerance are underway in our laboratory. A transformation system for *A. reptans* is being optimized and we envisage that excised leaves from transgenic plants, where GGT has been silenced, will provide key information to understanding the fundamental importance of these higher RFOs in frost tolerance.

## ACKNOWLEDGMENTS

The authors wish to thank Stefan Hörtensteiner for critical reading of the manuscript. This work was supported by a grant from the Swiss National Foundation.

## REFERENCES

Arakawa T., Prestrelski S.J., Kenney W.C. & Carpenter J.F. (2001) Factors affecting short-term and long-term stabilities of proteins. *Advanced Drug Delivery Reviews* **46,** 307–326.

Bachmann M. & Keller F. (1995) Metabolism of the raffinose family oligosaccharides in leaves of *Ajuga reptans* L. Inter- and intracellular compartmentation. *Plant Physiology* **109,** 991–998.

Bachmann M., Matile P. & Keller F. (1994) Metabolism of the raffinose family oligosaccharides in leaves of *Ajuga reptans* L. Cold acclimation, translocation, and sink to source transition: discovery of chain elongation enzyme. *Plant Physiology* **105,** 1335–1345.

Cacela C. & Hincha D.K. (2006) Monosaccharide composition, chain length and linkage type influence the interactions of oligosaccharides with dry phosphatidylcholine membranes. *Biochimica et Biophysica Acta* **1758,** 680–691.

Carpenter J.F. & Crowe J.H. (1989) An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. *Biochemistry* **28,** 3916–3922.

Chinnusamy V., Zhu J. & Zhu J.-K. (2007) Cold stress regulation of gene expression in plants. *Trends in Plant Science* **12,** 444–451.

Cunningham S.M., Nadeau P., Castonguay Y., Laberge S. & Volenec J.J. (2003) Raffinose and stachyose accumulation, galactinol synthase expression, and winter injury of contrasting alfalfa germplasms. *Crop Science* **43,** 562–570.

Gaffney S., Haslam E., Lilley T. & Ward T. (1988) Homotactic and heterotactic interactions in aqueous-solutions containing some saccharides – experimental results and an empirical relationship between saccharide solvation and solute–solute interactions. *Journal of the Chemical Society, Faraday Transactions* **84,** 2545–2552.

Hamman R.A. Jr, Dami, I.E., Walsh T.M. & Stushnoff C. (1996) Seasonal carbohydrate changes and cold hardiness of Chardonnay and Riesling grapevines. *American Journal of Enology and Viticulture* **47,** 31–36.

Haritatos E., Keller F. & Turgeon R. (1996) Raffinose oligosaccharide concentrations measured in individual cell and tissue types in *Cucumis melo* L. leaves: implications for phloem loading. *Planta* **198,** 614–622.

Hatanaka R. & Sugawara Y. (2008) Development of desiccation tolerance and vitrification in cultured cells of *Marchantia polymorpha*. *Cryobiology* **57,** 339.

Hincha D.K., Zuther E. & Heyer A.G. (2003) The preservation of liposomes by raffinose family oligosaccharides during drying is mediated by effects on fusion and lipid phase transitions. *Biochimica et Biophysica Acta* **1612,** 172–177.

Imanishi H.T., Suzuki T., Masuda K. & Harada T. (1998) Accumulation of raffinose and stachyose in shoot apices of *Lonicera caerulea* L. during cold acclimation. *Scientia Horticulturae* **72,** 255–263.

Inan Haab C.I. & Keller F. (2002) Purification and characterization of the raffinose oligosaccharide chain elongation enzyme, galactan : galactan galactosyltransferase (GGT), from *Ajuga reptans* leaves. *Plant Physiology* **114,** 361–371.

Keller F. & Pharr M. (1996) Metabolism of carbohydrates in sinks and sources: galactosyl-sucrose oligosaccharides. In *Photoassimilate Distribution in Plants and Crops: Source-Sink Relationships* (eds E. Zamski & A.A. Schaffer) pp. 157–183. Marcel Dekker, New York, NY, USA.

King R.W. & Zeevaart J.A.D. (1974) Enhancement of phloem exudation from cut petioles by chelating agents. *Plant Physiology* **53,** 96–103.

Klotke J., Kopka J., Gatzke N. & Heyer A.G. (2004) Impact of soluble sugar concentrations on the acquisition of freezing tolerance in accessions of *Arabidopsis thaliana* with contrasting cold adaptation – evidence for a role of raffinose in cold acclimation. *Plant, Cell & Environment* **27,** 1395–1404.

Koster K.L., Lei Y.P., Anderson M., Martin S. & Bryant G. (2000) Effects of vitrified and nonvitrified sugars on phosphatidylcholine fluid-to-gel phase transitions. *Biophysical Journal* **78,** 1932–1946.

Pennycooke J.C., Jones M.L. & Stushnoff C. (2003) Down-regulating $\alpha$-galactosidase enhances freezing tolerance in transgenic petunia. *Plant Physiology* **133,** 901–909.

Peters S., Mundree S.G., Thomson J.A., Farrant J.M. & Keller F. (2007) Protection mechanisms in the resurrection plant *Xerophyta viscosa* (Baker): both sucrose and raffinose family oligosaccharides (RFOs) accumulate in leaves in response to water deficit. *Journal of Experimental Botany* **58,** 1947–1956.

Prestrelski S.J., Tedeschi N., Arakawa T. & Carpenter J.F. (1993) Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. *Biophysical Journal* **65,** 661–671.

Salerno G.L. & Pontis H.G. (1989) Raffinose synthesis in *Chlorella vulgaris* cultures after a cold shock. *Plant Physiology* **89,** 648–651.

Santarius K.A. & Milde H. (1977) Sugar compartmentation in frost-hardy and partially dehardened cabbage leaf cells. *Planta* **136,** 163–166.

Shahba M.A., Qian Y.L., Hughes H.G., Koski A.J. & Christensen D. (2003) Relationships of soluble carbohydrates and freeze tolerance in saltgrass. *Crop Science* **43,** 2148–2153.

Tapernoux-Lüthi E.M., Böhm A. & Keller F. (2004) Cloning, functional expression, and characterization of the raffinose oligosaccharide chain elongation enzyme, galactan : galactan galactosyltransferase, from common bugle leaves. *Plant Physiology* **134,** 1377–1387.

Tapernoux-Lüthi E.M., Schneider T. & Keller F. (2007) The C-terminal sequence from common bugle leaf galactan : galactan galactosyltransferase is a non-sequence-specific vacuolar sorting determinant. *FEBS Letters* **581,** 1811–1818.

Turgeon R. & Gowan E. (1990) Phloem loading in *Coleus blumei* in the absence of carrier-mediated uptake of export sugar from the apoplast. *Plant Physiology* **94,** 1244–1249.

Valluru R., Lammens W., Claupein W. & Van den Ende W. (2008) Freezing tolerance by vesicle-mediated fructan transport. *Trends in Plant Science* **13,** 409–414.

Wiemken V. & Ineichen K. (1993) Effect of temperature and photoperiod on the raffinose content of spruce roots. *Planta* **190,** 387–392.

Zimmerman M. & Ziegler H. (1975) List of sugars and sugar alcohols in sieve-tube exudates. In *Encyclopedia of Plant Physiology* (eds M. Zimmermann & J. Milburn) pp. 480–503. Springer Verlag, Berlin, Germany.

Zuther E., Buchel K., Hundertmark M., Stitt M., Hincha D.K. & Heyer A.G. (2004) The role of raffinose in the cold acclimation response of *Arabidopsis thaliana*. *FEBS Letters* **576,** 169–173.

*Received 21 January 2009; received in revised form 30 March 2009; accepted for publication 1 April 2009*